Universidade de Aveiro Departamento de Biologia
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ECOLOGIA E BIOQUÍMICA EMBRIONÁRIA E LARVAR DE DECÁPODES DO MAR PROFUNDO

EMBRYONIC AND LARVAL ECOLOGY AND BIOCHEMISTRY OF OFFSHORE DECAPODS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Professor Doutor Henrique José de Barros Brito Queiroga, Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação científica da Doutora Antonina Maria de Melo dos Santos, Investigadora no Instituto Nacional de Recursos Biológicos – IPIMAR, Lisboa e Doutor Ricardo Jorge Guerra Calado, Investigador Auxiliar no Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Ácidos Gordos, Alimentação,Decápodes, Ao Largo, Desenvolvimento Embrionário, Distribuição Vertical, Enzimas Digestivas, Inanição, Investimento Maternal, Larva Invertebrada, Mar Profundo, Migração Vertical Diária, *Monodaeus couchi, Nephrops norvegicus*, Zoea.

resumo

Compreender a biologia das espécies de mar aberto é dificultado pela amostragem em ambiente de mar profundo. Além disso, condicionado pela vasta extensão do oceano aberto, o conhecimento da fase inicial de vida das larvas pelágicas é ainda relativamente limitado. Em espécies de decápodes bento-pelágicos, a transição da vida no fundo do mar para a coluna de água não só está associada a uma metamorfose drástica na morfologia, mas também a uma mudança de comportamento e de ecologia alimentar. O presente trabalho teve como objectivo principal investigar as adaptações fisiológicas, bioquímicas e comportamentais que ocorrem durante o desenvolvimento inicial em espécies de decápodes bento-pelágicos. Como organismos modelos foram usados o lagostim, *Nephrops norvegicus*, e o caranguejo *Monodaeus couchi*, pelo facto de ambas as espécies poderem ser capturadas ao largo da plataforma do NE Atlântico e a mais de 300 m de profundidade.

O Capítulo 1 apresenta os desafios enfrentados pelos adultos e larvas de decápodes que vivem nestes ambientes remotos, incluindo o efeito da disponibilidade de alimento no desenvolvimento, e dos processos oceanográficos na dispersão e recrutamento. Em particular, este trabalho pretende seguir a história de vida inicial, a começar com a variabilidade na composição dos ácidos gordos (AG) de embriões da mesma ninhada de *N. norvegicus* em desenvolvimento. Na maioria das fêmeas, não foram encontradas diferenças na composição dos AG de embriões alojados em ambos os lados da câmara de desenvolvimento embrionária. No entanto, todas as fêmeas apresentaram diferenças significativas nos perfis de AG de embriões situados em diferentes pleópodes. Possíveis causas para as variações registadas podem ser devidas ao investimento diferencial da fêmea durante a produção de ovócitos ou a mudanças no catabolismo dos AG durante o período de incubação promovido pela localização do embrião dentro da câmara de desenvolvimento embrionária. Em seguida foram investigadas em *N. norvegicus* as taxas de alimentação e a actividade de enzimas digestivas dos primeiros dois estádios larvares. Ambos os estádios foram capazes de maximizar a ingestão de alimento em situação de escassez de presas, e mostraram um aumento na taxa de alimentação depois de períodos de inanição. A actividade da amilase indicou que os hidratos de carbono não são a principal reserva de energia e que a alimentação pode ser obrigatória

logo após a eclosão para iniciar a actividade da amilase. A actividade da protease indicou que as reservas de proteínas são metabolizadas em inanição. Estes resultados indicam que na presença de maiores concentrações de plâncton as larvas podem maximizar a ingestão de presas e minimizar os efeitos prejudiciais provocados por intermitentes períodos prévios de fome, baixa densidade de presas, ou presas impróprias. Além disso, a mudança na actividade enzimática pode permitir às larvas recém-nascidas de *N. norvegicus* metabolizar as reservas de proteínas para sobreviver a curtos períodos de inanição.

De seguida foi estudado a influência das propriedades oceanográficas sobre a migração vertical das larvas. Em *M. couchi*, todos os estádios larvares apresentaram migração vertical nocturna inversa. A abundância dos dois primeiros estádios mostrou estar positivamente correlacionada com os níveis de clorofila a. A ocorrência de mudança ontogénica na distribuição vertical explicou os resultados; os primeiros estádios de zoeae permanecem na parte superior da coluna de água, uma zona rica em alimentos, ao contrário dos estádios posteriores que migraram para o fundo de forma a assentarem. O comportamento migratório vertical é susceptível de afectar a distribuição horizontal das larvas. Os padrões globais de circulação irão causar pequenas variações inter-anuais no recrutamento de larvas de decápodes, mas pequenas variações espacio-temporais, como eventos de afloramento, irão provocar alterações no padrão geral de dispersão.

resumo (cont.)

Ao longo do desenvolvimento, desde o embrião até a metamorfose para juvenil bentónico, os decápodes de mar aberto enfrentam muitos desafios. A sobrevivência do indivíduo em desenvolvimento depende significativamente da disponibilidade de alimento assim como das reservas fornecidas pela mãe. Apesar do comportamento de migração vertical permitir às larvas aproveitarem as variações de corrente para transporte, a influência dos padrões de circulação geral irá sobrepor-se às correntes locais e influenciar as condições de alimentação, a dispersão e o recrutamento.

keywords

Decapods, Deep-Sea, Diel Vertical Migration, Digestive Enzymes, Embryonic Development, Fatty Acid, Feeding Ability, Invertebrate Larva, Maternal Investment, *Monodaeus couchi, Nephrops norvegicus*, Offshore, Starvation, Vertical Distribution, Zoea.

abstract

Understanding the biology of offshore species is hardened by the difficulties of sampling in the deep-sea environment. Additionally, due to the vastness of the open ocean, knowledge of early life histories of pelagic larvae is still relatively scarce. In decapod species with bentho-pelagic lifestyle, the transition from life in the seafloor to the water column not only is associated with drastic morphological metamorphosis, but also with changes in behavior and feeding ecology. The purpose of the present thesis was to investigate physiological, biochemical and behavioral adaptation occurring during early development of such species. The Norway lobster, *Nephrops norvegicus*, and the crab *Monodaeus couchi* were used as a model as these two species are encountered off the NE Atlantic shelf at depth greater than 300 m.

Chapter 1 introduces the challenges faced by both adult and larvae inhabiting such remote habitats, including the effect of food availability on development and oceanographic processes on dispersal and recruitment. The thesis follows early life histories, starting with within-brood variability in the fatty acid (FA) profile displayed by developing *N. norvegicus* embryos. There were no differences in the FA composition of embryos sampled from both sides of the brooding chamber in most females. However, all females exhibited significant differences in the FA profiles of embryos sampled from different pleopods. Potential causes for the variations recorded may be differential female investment during oocyte production or shifts in FA catabolism during the incubation period promoted by embryo's location within the brooding chamber. Next, feeding rates and digestive enzymes activity of the early stage larvae was investigated in *N. norvegicus*. Both stages were able to maximize food intake when larvae were scarce and showed increased feeding rate following periods of starvation. Amylase activity indicated that carbohydrates are not the primary energy reserve and that feeding may be required soon after hatching to trigger amylase activity. Protease activity indicated that protein reserves are catabolized under starvation. These results indicate that larvae may maximize prey ingestion in the presence of plankton patches with higher food abundance and minimize the deleterious effects induced by previous periods of intermittent starvation or unsuitable prey densities/types.

Additionally, changes in enzymatic activity may allow newly hatched *N. norvegicus* larvae to metabolize protein reserves to overcome short-term starvation. Vertical migration behavior and the influence of oceanographic properties were studied next. All zoeal stages of *M. couchi* displayed reverse diel vertical migration. Abundance of early stages was correlated with chlorophyll a levels. An ontogenic shift in vertical distribution explained the results; earlier zoeal stages remain in the food-rich upper water column while later stages migrate to the bottom for settlement. This vertical migration behavior is likely to affect horizontal distribution of larvae. Indeed, global current patterns will result in low inter-annual variations in decapod larvae recruitment, but short term variations such as upwelling events will cause deviation from the expected dispersal pattern.

Throughout development, from the embryo to metamorphosis into benthic juvenile, offshore decapods face many challenges. For the developing individual survivorship will depend heavily on food availability but also on the reserves passed on by the mother. Even though vertical migration behavior can allow the larvae to take advantage of depth varying currents for transport, the effect of general circulation pattern will superimpose local current and influence feeding conditions and affect dispersal and recruitment.

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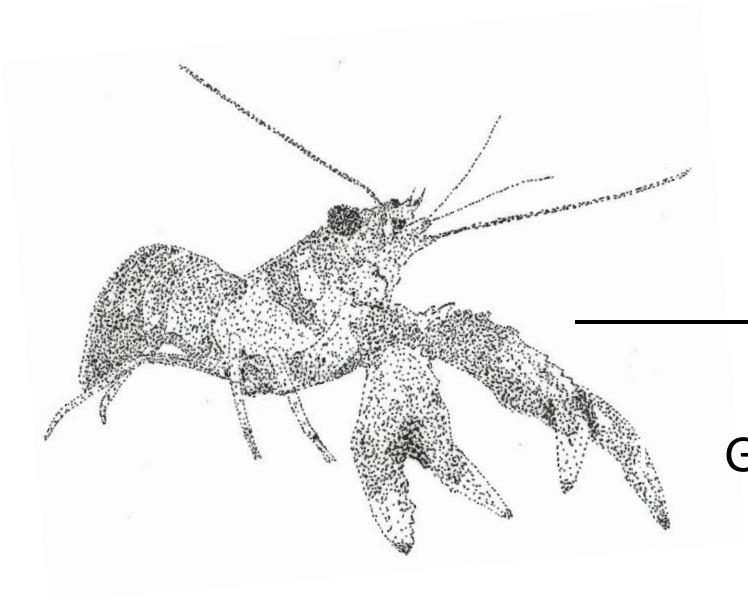

Chapter 1

General Introduction

Embryonic and larval stages are important phases of decapod crustaceans life cycle. It is widely accepted that pre-settlement processes have an important impact on metamorphosis and survival of post-larvae and juveniles (Giménez 2010). Until recently, most studies focused separately either larval or post settlement processes. However, as stressed by Pechenik (2006), metamorphosis should not be considered as a new beginning. A carry-over effect from earlier larval history will affect post-settlement performance of newly settled juvenile (Giménez 2006). Similarly, the quality of maternal investment into the embryo or variations in environmental conditions during embryonic development will ultimately affect larval development and survival (Giménez and Anger 2003). Many studies have documented the existence of inter-population differences in embryonic and larval development (e.g.: Bas et al. 2007; Kunisch and Anger 1984; Ouellet and Plante 2004; Rotllant et al. 2004). For instance, in species with a wide distribution, geographic differences in environmental conditions strongly influences early life history processes (Brante et al. 2003; Rotllant et al. 2004). Similarly, species displaying extended reproductive seasons and/or occupying habitats exposed to strong environmental gradients may experience variations in food availability, temperature and/or salinity, which may result in intra- and inter-population variability in the energetic content of embryos and newly hatched larvae (e.g.: Brante et al. 2003; Giménez and Anger 2003; Kunisch and Anger 1984; Tuck et al. 2000; Verísimo et al. 2011). On smaller spatial scales, local environmental conditions might not be identical for all developing siblings and as a result, within-brood variability will be observed. In this sense, an increased within-clutch variability can reduce the variation in reproductive success among generations, by ensuring that at least some offspring are likely to have the suitable phenotype for the local environment (Crean and Marshall 2009).

The extent to which latent effects of embryonic and/or larval development regulate the populations of coastal invertebrates is still largely unknown (Pechenik 2006). Nevertheless, as stated above, within-brood variability resulting from differential maternal investment into embryonic reserves or larval experience will affect larval processes and/or metamorphosis. Because of the critical role played by the larval phase in dispersal, factors affecting larval traits will impact recruitment and therefore

affect population dynamics (Giménez 2006, 2010). The purpose of this thesis was to investigate early life history processes of offshore benthic decapods: 1) document inter-individual and within-brood variability in embryonic reserves, 2) investigate early larval behavioral and physiological responses to changes in food quality and quantities, and 3) describe larval migratory behaviors and dispersal. Two species were used in this thesis to serve as models for offshore benthic Pleocyemata decapods, the Norway lobster *Nephrops norvegicus* and the xanthid crab *Monodaeus couchi*.

In this chapter it will be identified where, in the life cycle of benthic decapod, differences in environmental conditions might result in within-brood variability in early life development and survival. A summary of current knowledge about the reproductive and early life history of offshore benthic decapod species is given. Challenges faced by females will be briefly overviewed, focusing on phases playing a crucial role in the reproduction process, mainly ovogenesis. Essential factors for successful larval development to juvenile, such as embryonic reserves, environmental conditions and food quality and quantity, as well as, recruitment and settlement processes, will also be assessed. Finally, the biology and ecology of the two model species will be briefly reviewed.

1.1 Ovarian and embryonic development in benthic offshore Pleocyemata decapods

Due to the difficulties existing in sampling the benthic offshore environment, the current knowledge on the biology of species from this habitat is far from complete. Except for chemoautotrophic processes taking place at hydrothermal vents and cold seeps (Gage and Tyler 1991), owing to the lack of sunlight on the sea floor of the open ocean and, therefore photosynthesis, food is provided to the benthic community through sedimentation from upper layers. As a result, the availability of food resources for the deep benthos largely depends on the flux of particulate organic carbon from the epipelagic zone, although there are time lags involved (Company et al. 2003; Fanelli and Cartes 2004; Johnson et al. 2007). Consequently, the majority of the offshore benthic species are deposit feeders, or complete their predator diet through

scavenging. Chitinous exoskeleton shed by planktonic organisms represent a reliable, constant food sources (Johnson et al. 2007), while carcasses of larger organism such as squids or whales constitute a large but sporadic supply of lipids and proteins (Kemp et al. 2006). Nevertheless, on the way down, sinking particles, mostly originating from plant or animal (e.g.: feces, dead individual, molt, etc…), are grazed upon by pelagic scavengers and therefore lose much of their nutritive qualities before reaching the benthos, a loss that increases proportionally with increasing depth (Gage and Tyler 1991). Food is therefore the most limiting factor for secondary production in this environment (Stockton and DeLaca 1982).

Many adult decapods have a benthic existence. Those that live further offshore will therefore live deeper and face many challenges related to the deep sea environment. As discussed above, food is the most limiting factor for organisms inhabiting offshore benthic habitats (Gage and Tyler 1991; Labropoulou and Kostikas 1999). Unlike decapod species from shallow waters, offshore species cannot rely solely on predation for survival and most are expected to display primarily a scavenging lifestyle (Fanelli and Cartes 2004). Nevertheless, dietary habits are fairly unknown due to the difficulties of sampling, either through direct observations or stomach content analysis, as the gastric mill of decapod generally grinds preys beyond identification (Gage and Tyler 1991). Additionally, food availability is bound to influence the biology of offshore benthic species, especially during the critical periods of their life history (*e.*g. molt, gamete production, egg incubation…). Indeed, the synchrony between the reproductive cycle and seasonal phytoplankton blooms in surface waters has already been documented in deep-sea decapods (Copley and Young 2006; Hilario et al. 2009; Perovich et al. 2003). Additionally, shifts in food resources influencing growth have been shown to affect ovarian composition as well (Tuck et al. 1997b).

Plasticity in maternal investment is often illustrated by the variability observed in inter-individual and/or within-brood egg size (e.g.: Bas et al. 2007; Briones-Fourzan et al. 2009), which, despite some small limitations, can ultimately be used to assess embryonic energetic input by the female (Moran and McAlister 2009). In the Pleocyemata, egg production is as follows: vitellogenesis is divided into two phases; first yolk is deposited slowly into the oocytes which gradually increase in size; second,

large amount of yolk is deposited in the oocytes over a short period of time. The quality of nutrients deposited into the developing oocyte during this second phase is more likely to be affected by the feeding status of the female. Besides the ovaries, nutrients used during vitellogenesis in decapod can originate from various sources, including the hepatopancreas, hemocytes and subepidermal adipose tissue (Talbot and Helluy 1995). After ovulation, the mature oocytes are spawned and fertilized either internally or externally depending on the species. After fertilization, embryos become attached to the ovigerous setae of female's pleopods until their development is complete and hatching occurs. During that period, the female cares for the embryos by fanning the pleopods, bringing water and thus fresh oxygen to the egg mass. Parasites, as well as unhealthy and dead eggs, are also periodically removed through grooming.

In marine invertebrates, maternal investment is a major factor in shaping population structure and dynamics (Hammerschmidt et al. 2011; Marshall and Keough 2008). Because embryonic development in decapods is completely lecithotrophic (Anger 2001), the quality and quantity of embryonic reserves provided by females are critical and impact development, hatching and early larval survival (Giménez 2006). The resources available to the embryo are those that have been provided by the female, which are influenced by the maternal diet prior to ovogenesis (Racotta et al. 2003). As a result, gonadal maturation in decapods has large associated energy costs (Rosa and Nunes 2002). By understanding the biochemical shifts occurring during embryonic development, it is possible to identify the nutritional requirements exhibited by developing embryos. Additionally, changes in nutrient allocation may help understand how within-brood variability will ultimately affect larval survival. Proteins are known to be the main components of marine invertebrate eggs (Holland 1978). However, lipids play a central role in the embryonic metabolism, since they represent the most important energy source and form at least 60% of the total energy expenditure of developing crustacean embryos (Wehrtmann and Graeve 1998). Nevertheless, for planktotrophic larvae in the open ocean, food may not be available right after hatching and, when facing starvation, the only energetic reserves allowing survival are of embryonic origin (Anger 2001; Rotllant et al. 2001). In this sense, the quantity and

quality of nutrients remaining from embryonic development will highly influence larval development and survival, and ultimately affect population dynamics.

Alternatively, differences in environmental conditions within the brooding chamber may promote a shift in nutrient consumption during the incubation period. Variable oxygen concentration within the dense embryo mass has already been described for brachyuran crabs (Fernandez et al. 2003). Embryonic metabolic rates commonly increase in reduced oxygen environment and yolk reserves provided by the female are depleted at a faster rate (Brante et al. 2003). This scenario will cause a differential catabolism of embryonic reserves and eventually lead to asynchronous larval hatching (Eriksson et al. 2006; Fernandez et al. 2003). Indeed, decapods have been recorded to hatch their larvae over several consecutive nights (Pandian 1970). These episodes were often considered as a "laboratorial artifact". Nevertheless, regardless of the proximate cause, within-brood variation in embryonic nutrients may result in asynchronous embryonic development, and may ultimately result in asynchronous hatching of larvae with variable nutritional reserves (Wickins et al. 1995). This variability at birth will, in turn, affect larval success in recruitment to suitable juvenile habitat.

1.2 Life history processes of Pleocyemata decapod larvae in offshore environments

Due to its harsh environmental conditions and patchy food distribution, the open ocean habitat is not optimal for larval development (Giménez and Anger 2005). In offshore benthic decapods, the depth where larval hatching takes place may also play a role in food availability. In larvae hatching below the photic zone, prey items may not be sufficient to fulfill the nutritional needs of the first larvae stage. In fact, it is possible that suitable prey may only be available once these larvae reach the upper regions of the water column (Labropoulou and Kostikas 1999). After hatching, decapod larvae are known to migrate vertically to the photic zone (Queiroga and Blanton 2005). This aspect of decapod's early life cycle may translate into a potential period of suboptimal feeding, or even post-hatching starvation. Following the first vertical migration, natural patchiness of the pelagic environment will result in variable food conditions (Andersen and Nielsen 2002; Folt and Burns 1999; Pinel-Alloul 1995) and feeding larvae have

evolved mechanisms allowing them to cope with those trophic mismatches which will be discussed below.

After a period of lecithotrophic embryonic development, the eggs of decapods will hatch into free-swimming larvae. Species of deep sea habitats tend to invest more energy into each individual embryo, and therefore tend to produce a smaller number of larger eggs (Anger 2001; Gage and Tyler 1991). The number of molts necessary to reach post larval stage greatly varies intra-specifically but again the number of larval stage and larval duration have the tendency to decrease with increasing depth of adult habitat (Anger 2001). Additionally, the dependence on food will also decrease with depth. These strategies will translate into reduced mortality and increased recruitment and settlement success (Gage and Tyler 1991).

1.2.1 Feeding

Regardless of larval geographical range or hatching period, it is widely accepted that planktonic larvae are naturally subjected to intermittent periods of starvation and/or suboptimal prey availability due to the natural patchiness of plankton distribution in the oceanic environment (Andersen and Nielsen 2002; Folt and Burns 1999; Pinel-Alloul 1995). Studies have highlighted how, in decapods, early larval feeding greatly affects their development, since these organisms may not recover from extended period of nutritional stress even after feeding resumes (e.g.: Anger et al. 1985; Calado et al. 2010; Giménez and Anger 2005; Pechenik 2006; Rotllant et al. 2001). The larvae of species hatching in regions of low food availability have developed morphological (Fenaux et al. 1994; Strathmann et al. 1993) or behavioral (McConaugha 2002) adaptations to enhance their overall feeding efficiency. Planktotrophic larvae of many benthic species appear to have evolved a certain feeding plasticity, in order to be able to feed over a broad range of prey sizes, types and abundances (Hinz et al. 2001; McConaugha 2002; Perez and Sulkin 2005; Strathmann and Bone 1997). By feeding on smaller and/or suboptimal food items, developing larvae may minimize the negative effects of low food density and avoid starvation. In fact, this feeding plasticity had already been previously documented in decapods, such as homarid lobsters and

brachyuran crabs that were recorded to successfully perform suspension feeding (in contrast with the most commonly recorded raptorial feeding behavior) when subjected to suboptimal food conditions(Barshaw and Bryant-Rich 1988; McConaugha 2002).

1.2.1.1 Larval nutrition and digestion

A prey item captured by a feeding decapod larva will be first masticated by the mandibles. While food is processed through the esophagus and cardiac stomach some mechanical digestion takes place. Upon reaching the gastric mill food is broken down further through mechanical grinding and internal mastication. In the pyloric stomach, the gastric juices will then be filtered and transferred to the hepatopancreas, where intracellular digestion will be catabolized by digestive enzymes. Fecal pellets will finally be expelled through the anus (Anger 2001). During this process, nutrients important for larval survival and development are assimilated and transformed.

In decapods, essential nutrients include lipids, proteins and carbohydrates. Lipids are probably the most important energy source (Anger 2001) and are catabolized when larvae are deprived of food (Ritar et al. 2003a). During starvation, proteins, along with lipids, play an important role as energy sources to the developing larva (Anger 2001; Johnston et al. 2004). Despite the fact that marine decapods are not able to efficiently store carbohydrates reserves and that lipids and proteins may be more relevant to fuel larval energetics, carbohydrates are expected to be important, even though its role is not yet fully understood (Sánchez-Paz et al. 2006).

Despite ample evidences that larvae of most species of decapods are omnivorous (e.g.: Barshaw and Bryant-Rich 1988; Hinz et al. 2001; McConaugha 2002; Perez and Sulkin 2005), larvae are not necessarily adapted to digest the various nutrients ingested with the same efficiency. The effectiveness of food assimilation primarily depends on two factors: 1) gut evacuation time (GET, the interval between first consumption of an item and its first appearance in fecal material) and 2) activity of digestive enzymes. In many species there is a negative relationship between the two factors, enzymatic activity

increasing with decreasing GET. Indeed, increased residence time of food within the gut implies higher assimilation efficiency and as a result high enzymatic activity is not necessary (Kurmaly et al. 1990).

During digestion, the activation of specific enzymes, primarily synthesized in the hepatopancreas, will result in the degradation of different dietary macromolecules. The analysis of their dynamics during periods of starvation (or suboptimal feeding) may be an excellent indicator of which nutrients act as energy reserves and which are preferentially catabolized under these scenarios (Johnston 2003; Johnston et al. 2004; Jones et al. 1997; Kamarudin et al. 1994; Lovett and Felder 1990a; Rotllant et al. 2010). For instance, high protease activity is indicative of protein catabolism, while amylase activity indicates the consumption of carbohydrates (Johnston 2003; Kamarudin et al. 1994). Additionally, it has been suggested that amylase may play other role than just catabolizing carbohydrates, even though its role has not yet been fully understood (Jones et al. 1997). Studying amylase activity in omnivorous species may help understand why this enzyme is active in spite of the scarcity of its substrate.

1.2.1.2 Feeding strategies

Most decapod larvae require food to successfully develop and are thus classified as planktotrophic. On the other end of the continuum, there are species which larvae can accomplish their complete (or partial) larval development, without feeding. These lecithotrophic larvae rely entirely on internal nutritional reserves that have been provided from the female to the embryo. This mode of development is often associated with environments where food availability is highly unpredictable, such as the deep sea (e.g.: Pond et al. 1997). However, even in areas that commonly display high food production, larvae can be subjected to variable periods of food limitation due to the natural patchiness of the pelagic environment (Andersen and Nielsen 2002; Folt and Burns 1999; Pinel-Alloul 1995; Strathmann 1985).

Larval dependence on food may vary intra-specifically depending on developmental stage. The ability of newly hatched larvae to successfully molt through one or more

larval stages without feeding is called primary lecithotrophy. The amount of time a larva will be able to survive starvation and still be capable of normal development once suitable food conditions are encountered will depend upon the energetic reserves provided by the female into the embryos. During the planktonic larval phase, some species will be able to start feeding when food becomes available (facultative lecithotrophy) while others are unable to ingest food even if preys are encountered (obligate lecithotrophy) (Anger 2001).

As in many species the yolk reserves are highly depleted at hatching, newly hatched larvae of many species are not able to successfully reach the second larval stage relying solely on the nutrients provided by the female. These larvae will therefore be dependent on exogenous food, whose quality and quantity will influence larval development. However, energetic reserves stored from exogenous feeding may allow later stages to develop without food through metamorphosis (e.g.: Abrunhosa et al. 2008; Anger and Schubart 2005; Thessalou-Legaki et al. 1999). This case of endotrophic development is called secondary lecithotrophy and can be explained in post-larval stage as an adaptation to food limitation as the individual is searching for a suitable habitat to metamorphose and settle as a benthic juvenile (Calado et al. 2007b; Calado et al. 2010).

These different feeding strategies have evolved in response to different food quantity and quality available to both adults and larvae in the various habitats occupied by decapods, and maximize larval survival and development. However, regardless of the strategy, unpredictable food shortage may occur at any point of the life cycle and different mechanisms exist to allow the planktotrophic larvae to cope with periods of starvation. These responses include changes in feeding rate and reduced prey selectivity as the larvae become increasingly opportunistic under reduced food conditions (Barshaw and Bryant-Rich 1988; McConaugha 2002). In addition, gut evacuation time and/or enzymes activity can increase under such unfavorable conditions to optimize the assimilation of the limited amounts of nutrients ingested when the larvae encounters areas of unsuitable prey quantity and quality (Lovett and Felder 1990a).

1.2.2 Migrations

Vertical migration is a behavior commonly observed in marine organisms which can enhance feeding, predator avoidance or transport. Since most decapod larvae have limited swimming abilities, vertical swimming generally represents the only form of actively influencing horizontal dispersal (Sulkin 1984). Vertical position of offshore decapod larvae is known to vary according to the phase of the day, as well as to ontogenic development. However, regardless of the type, vertical migration ultimately affects horizontal transport by subjecting larvae to depth varying current intensities and directions (dos Santos et al. 2008; Queiroga and Blanton 2005).

1.2.2.1 Diel vertical migrations

The most commonly known and described pattern of zooplanktonic migration takes place on a daily cycle. Diel vertical migration (DVM) behavior displayed by decapod larvae conform to the three main patterns described for general zooplanktonic forms (Forward 1988). In nocturnal migration, which is the most common form, larvae rise to surface water during the night to feed and sink below the photic zone during the day, probably to reduce predation pressure (Forward 1988; Pearre 2003). In the second form, known as reverse diel vertical migration, larvae rise to the surface during the day and sink to deeper water at night (Forward 1988). Finally, in twilight migration, larvae reach minimum depths near sunset, descend to intermediate depths during the night and rise again to surface near sunrise before sinking to remain in deeper water during the day (Forward 1988). Nevertheless, even within the same species, DVM behavior is not always the same and might vary with area (*e.g.* distance from coast) or environmental conditions such as fluctuations in predator or prey abundances or vertical stratification (Han and Straskraba 2001). Reverse DVM has been suggested as a defense against predator undergoing nocturnal vertical migration. As observed in various copepod species, when predators exhibit nocturnal DVM their prey may undergo reverse DVM (Irigoien et al. 2004; Osgood and Frost 1994). This behavior has been associated to areas where the predators present rely more on tactile stimuli than sight, like it is the case for many invertebrate predators (e.g.: chaetognaths and

gelatinous zooplankton) which themselves are undergoing nocturnal DVM as a way to escape fish predators (Tester et al. 2004). Additionally, reverse DVM has also been observed in areas with lower abundances of predatory fish (Lagergren et al. 2008). Since shifts in predator populations occur, the prey must be able to adjust their behavior accordingly, and larvae of some species might be able to exhibit plasticity in their DVM behavior with changing environmental conditions.

Knowledge of DVM behavior of larvae of species that accomplish their entire lifecycle offshore is limited to a few species. Nocturnal diel vertical migration has been observed in *Cancer* spp. and *Randalia ornata* zoeae, but megalopae did not display clear vertical migration, suggesting that these species undergo an ontogenetic shift in vertical migration behavior off the coast of California (Shanks 1986).

1.2.2.2 Ontogenic migration

On a longer time scale, vertical position in the water column will vary with development stage. Newly hatched larvae feeing in surface waters will be encountered at lower depth than the megalopae searching for suitable settlement site near benthic adult habitats (Queiroga and Blanton 2005; Queiroga et al. 2007). This shift results in changing behavioral responses during ontogenetic development (Queiroga and Blanton 2005; Queiroga et al. 2007). This behavior has been observed in many decapods accomplishing their entire life cycle offshore, including prawns, such as *Aristeus antennatus* (Carbonell et al. 2010), and brachyuran crabs such as *Geryon quinquedens* (Kelly et al. 1982). In early stages the combination of high barokinesis and negative geotaxis acts to position the larvae in a food-rich environment close to the surface, hence favoring growth (Sulkin 1984). In contrast, the megalopa is the stage that needs to encounter a suitable environment for settlement and subsequent metamorphosis. Therefore, it will move towards the bottom, which is usually achieved by positive geotaxis. In general terms, the response of larvae to a number of environmental factors involved in depth regulation, such as light, gravity or pressure, changes through ontogeny, resulting in deeper distributions in later stages (Sulkin 1984). Additionally, changes in morphological and biochemical adaptations of larvae

throughout ontogeny also affect their buoyancy, further influencing their vertical distribution (Sulkin 1984).

1.2.3 Dispersal and recruitment

While DVM behavior can be related to predator-avoidance mechanisms (Han and Straskraba 2001), it ultimately affects horizontal transport by subjecting larvae to variable current intensities and directions (dos Santos et al. 2008; Queiroga and Blanton 2005). It is less clear, however, how vertical migrations in the open ocean result in retention in, or transport to, areas more favorable for successful development and settlement.

It is however widely documented that biotic and abiotic cues influence larval position in the water column. Examples of such factors include: physical properties of the water (*i.e.* temperature, salinity, and hydrostatic pressure), light levels, predator and prey concentration, state of feeding, and endogenous rhythms (Epifanio and Garvine 2001; Folt and Burns 1999; Forward 1988). Larvae not only respond to exogenous cues to be transported to suitable settling habitat, but also need to be retained in water masses with tolerable conditions for development (Hobbs et al. 1992). For instance, there is a negative relationship between temperature and larval development time (O'Connor et al. 2007), which will affect mortality because an extended larval development period at low temperatures translates into a larger time period of exposure to mortality factors such as predation (Morgan 1995). Additionally, extreme conditions of temperature will increase mortality (Anger 1987) and many decapod species show enhanced development under typical oceanic salinity (approximately 35) (Anger et al. 2000; Anger et al. 1998; Baylon and Suzuki 2007). Decapod larvae therefore detect and respond to changes in those physical parameters so that they are retained in a specific water mass favorable to development and thus greatly affecting transport, survival and settlement (Forward and Tankersley 2001; Shanks 1986).

1.3 Model Species

Both *Nephrops norvegicus* and *Monodaeus couchi* may provide a good model for the understanding of larval ecology of deep-sea decapod crustaceans. The observations of the larvae of these species may help expand our understanding on the general factors that control the spatial and temporal distribution of offshore benthic decapod crustacean larvae in general.

1.3.1 *Nephrops norvegicus*

The Norway lobster, *Nephrops norvegicus* (Linnaeus, 1758), is a commercially important benthic decapod crustacean commonly found in Northeastern Atlantic waters, from the Coast of Iceland to Morocco, and in the Mediterranean Sea (d'Udekem d'Acoz 1999). The adult depth range varies from 15 to 800 m, although they are typically found on the NE Atlantic shelf between 300 and 600 m depth (Tuck et al. 1997a) and 200 and 800 m in the Mediterranean (Maynou and Sardà 1997). Off the coast of Portugal, the adults are thus encountered at a depth (between 400 to 800 m) where food availability is reduced. Since lipid requirements of developing ovary seem to be more dependent on the recent ingestion of dietary lipids than on hepatopancreas reserves (Rosa and Nunes 2002), the state of feeding during ovogenesis will play an important role in energy allocation into the oocytes. Indeed, variations in nutrient content of the ovaries has been observed to vary between populations (Tuck et al. 1997b). Rosa el al. (2003) also extensively described fatty acid consumption through embryonic development. Lipid catabolism plays a crucial role during development, and up to 70% of the initial total lipid content can be depleted before hatching (Rosa et al. 2005; Rosa et al. 2003). In this way, it is possible that female's feeding status shortly at vitellogenesis may play a decisive role in hatching success and larval quality.

The reproduction period of this species varies with location, as average embryo incubation period lasts up to 10 months in the Northeastern Atlantic and only 6 months in the Mediterranean Sea (Sardà 1995). These latitudinal differences affect the

timing of larval hatching in *N. norvegicus* , with larval release occurring by early spring in the Northeastern Atlantic and at the end of winter in the Mediterranean (Rotllant et al. 2004). During such an extended period of incubation, variation in environmental conditions can greatly affect embryonic metabolism and therefore the use of yolk reserves. Potential deleterious effects resulting from suboptimal environmental conditions are commonly reduced through adaptations in the brooding behavior of ovigerous females (Eriksson et al. 2006). However, despite maternal care, differences in nutritional state have already been recorded in newly hatched *N. norvegicus* larvae. In the Mediterranean, larvae are larger in size and richer in lipids and proteins than those from the Irish sea, which emphasizes the major role that these features may play for larval survival in oligotrophic environments (Rotllant et al. 2004).

Among the early works on *N. norvegicus*, many were made with females from Portuguese waters such as the studies by Figueiredo who described population structure of this species off the coast of Portugal (Figueiredo and Thomas 1967) and provided a general microscopical description of the ovaries (Figueiredo 1972) and established a color based scale to visualize ovarian development (Figueiredo and Barraca 1963). Figueiredo and Nunes (1965) also estimated that egg loss during incubation off the coast of Portugal was of about 75% regardless of the size of the female. Additionally, in the first steps on culturing Norway lobster larvae in the laboratory, Figueiredo and Vilela (1972) who found that the best temperature level for incubation and survival were between 11 and 14ºC.

Off the Coast of Portugal, adult Norway lobsters occur at depths ranging from 400 to 800 m and larval hatching period is known to take place from December to April (dos Santos and Peliz 2005). After incubation on the female abdomen, larvae hatch as planktonic larvae, develop through 3 larval stages and then migrate back near the bottom during the decapodite stage before settling on the benthos as a juvenile (Sardà 1995). Dos Santos and Peliz (2005) established that newly hatched larvae escape oligotrophic environment in the aphotic zone by undergoing a migration towards the food rich photic zone near surface to forage for suitable prey.

1.3.2 *Monodaeus couchi*

Monodaeus couchi is a xanthid crab that inhabits muddy substrates (Ingle 1983b) from England to Angola and in the Mediterranean over a bathymetric range extending from 60 to 1300 m (Gonzalez-Gurriarán and Méndez G. 1985; Zariquiey Alvarez 1968). It is commonly associated with mud volcanoes, carbonate chimneys and cold seeps in the Gulf of Cadiz region (Cunha, M. R., University of Aveiro, unpublished observations). In slope habitats of Southern Iberian Peninsula and of the Mediterranean Sea the species co-occurs with the commercially important Norway lobster *Nephrops norvegicus* (Gonzalez-Gurriarán and Méndez G. 1985; Maynou and Sardà 1997; Mori et al. 1995; Tuck et al. 1997a). After hatching the larvae undergoes 4 zoeal stage and one megalopal stage (Ingle 1983a). So far, the biology of this species has not been studied.

1.4 General objectives and thesis outline

In order to better understand the early life biology of offshore species with a benthopelagic lifecycle in offshore habitats, a series of studies were performed using decapod species as a model. Individual variability of maternal input into embryos, early larval behavior, feeding and digestion, as well as general decapod larval distribution of the Southwestern coast of Portugal, were investigated. More specifically, the objectives of this thesis were to:

- Investigate inter-individual variability and document the existence of within-brood variation in the fatty acid profiles of *Nephrops norvegicus* embryos,
- Clarify the behavioral and digestive enzyme response of early-stage *Nephrops norvegicus* larvae under various feeding scenarios,
- Describe both diel and ontogenic vertical migration behavior of *Monodaeus couchi* throughout larval development,
- Obtain data on the composition, abundance and distribution of decapod crustacean larvae off the south Portuguese coast during winter.

The following provides the content of each chapter. Each one, except the introduction and concluding remarks, represents different units with specific and well defined objectives, organized in individual sections (Introduction, Methods, Results, Discussion and References).

The quality and quantity of maternal nutrient investment to the embryos will highly affect larval success. In **Chapter 2**, the existence of within-brood variability in the embryonic fatty acid (FA) profile was investigated across the brooding chamber to determine if attachment site affected FA profiles of developing *N. norvegicus* embryos. In general, FA composition of embryos sampled from both sides of the brooding chamber did not differ according to that factor. In contrast, all females exhibited significant differences in the FA profiles of embryos sampled from different pleopods. Overall, saturated FA (SFA) profiles were more variable across the brooding chamber than highly unsaturated FA (HUFA). Potential causes for intra-individual variations recorded in FA profile may be differential female investment during oocyte production and/or shifts in FA catabolism during the incubation period promoted by embryo's location within the brooding chamber.

- **Pochelon P. N.**, H. Queiroga, T. Lopes da Silva, A. Reis, A. dos Santos & R. Calado. Inter-individual and within brood variability in the fatty acid profiles of Norway lobster, Nephrops norvegicus (L.) embryos. Submitted to Marine Biology

After hatching, and independently from maternal investment, planktotrophic larvae might face intermittent periods of starvation due to the natural patchiness of the ocean. **Chapter 3** investigated the feeding response of the first two zoeal stages of *N. norvegicus* under variable prey densities, prey types, feeding histories and photoperiods. Both zoeae (Z) I and II increased the number of consumed prey with increasing food levels. Both stages displayed preference in prey size, as ZI preferred smaller preys and ZII displayed higher ingestion rates of larger preys at higher food concentrations. Feeding history also affected prey ingestion which indicated that larvae may maximize prey ingestion in the presence of plankton patches with higher

food abundance and minimize the deleterious effects induced by previous periods of intermittent starvation or unsuitable prey densities/types.

- **Pochelon P. N.**, R. Calado, A. dos Santos & H. Queiroga. 2009. Feeding Ability of Early Zoeal Stages of the Norway Lobster *Nephrops norvegicus* (L.). Biological Bulletin. 216: 335–343.

In addition to feeding plasticity, adaptations in digestive mechanisms can further enhance the ability of larvae to cope with intermittent starvation and/or variations in prey quality. In **Chapter 4**, protease and amylase activity of *N. norvegicus* was investigated in early stage larvae under different feeding scenarios. Amylase activity was generally low, indicating that carbohydrates are not used as the primary energy reserve and that feeding is required soon after hatching to trigger amylase activity. In contrast, protease activity increased in starved larvae after more than 12h of starvation, indicating that protein reserves are being catabolized. These highlight the role of early feeding plasticity in enzymatic activity of *N. norvegicus* larvae to overcome short-term starvation.

- **Pochelon P. N.**, H. Queiroga, G. Rotllant, A. dos Santos & R. Calado. Effect of unfavorable trophic scenarios on amylase and protease activity of *Nephrops norvegicus* (L.) larvae during their first vertical migration: a laboratory approach. In press, Marine Biology.

Knowledge of larval distribution and abundance is of major importance to predict the location and size of a breeding population. **Chapter 5** describes vertical distribution of the larvae of a brachyuran crab, *Monodaeus couchi*. Abundance and distribution of ZI and II were correlated. For all stages, abundance decreased with depth during the light phase while it increased with depth at night, thus displaying reverse DVM. Additionally, this species undergo an ontogenic shift in vertical distribution as earlier zoeal stages remain in the upper layers while later stages were encountered deeper.

- **Pochelon P. N.**, A. dos Santos, A. M. P Santos & H. Queiroga. Vertical larval distribution of an offshore brachyuran crab, *Monodaeus couchi*, off the South Coast of Portugal. In review, Marine Ecology Progress Series.

More generally, **Chapter 6** assessed spatial distribution and abundance of the decapod larvae in the Southwestern Iberian coast for two consecutive years. Coastal species that dominate in the first campaign were almost completely absent the following year and annual differences in overall abundances were observed. Additionally, distribution and abundance varied between sampling area, which is likely to be the result of general circulation patterns. An upwelling event that occurred off the southwest coast of Portugal at the time of sampling explained the differences in decapod larvae abundances and diversity observed between the Southwestern and Northeastern part of the sampling area. In the NE, the most abundant species were retained onshore while offshore transport occurred in zooplankton and to a lesser extent in some shelf species.

- **Pochelon P. N.**, A. dos Santos, J. Dubert, R. Nolasco, A. M. P Santos, & H. Queiroga. Species composition and distribution of decapod larvae off the South Coast of Portugal. In review, Journal of Sea Research.

Chapter 7 recapitulates the main finding of the separate data chapters and the significance of these results for understanding reproduction, development and survival of benthic offshore decapods. The different challenges faced and how each stage has evolved to cope and/or overcome them is discussed. Finally, this chapter includes suggestions of future directions for offshore decapod research that will allow a better understanding of how suboptimal conditions during early life history affect later stages. Important characteristics that should be considered when choosing suitable model species are also discussed.

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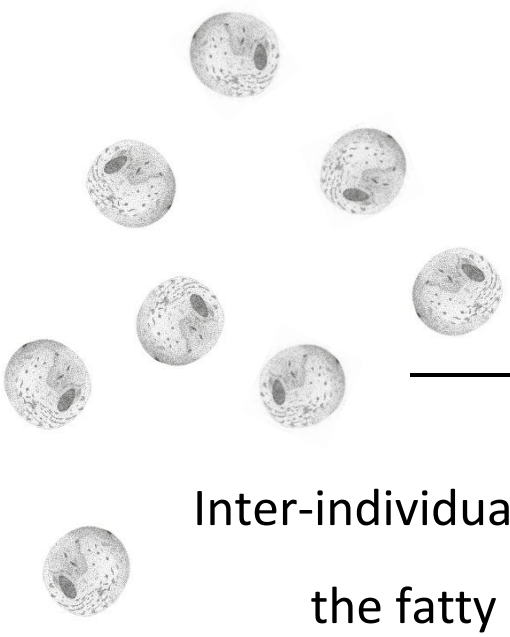

Chapter 2

Inter-individual and within brood variability in the fatty acid profiles of Norway lobster, *Nephrops norvegicus* (L.), embryos.

In review; Marine Biology

Chapter 2

Inter-individual and within brood variability in the fatty acid profiles of Norway lobster, *Nephrops norvegicus* (L.), embryos

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2.1 Abstract

The present study investigated the existence of inter-individual and within-brood variability in the fatty acid (FA) profile of developing embryos of *Nephrops norvegicus*. In all surveyed females (n=5), the quantitatively most important FA were: 22:6*n*-3 (20.8±3.9% average of total FA ± standard error), 18:1*n*-9 (19.5±2.0%), 16:0 (15.2±3.4%), 20:5*n*-3 (10.2±1.4%), 16:1*n*-7 (8.9±1.6%), and 18:1*n*-7 (5.7±1.3%). Differences in FA profiles of embryos in the same clutch were assessed using brooding chamber side (left and right) and pleopod (1^{st} & 2^{nd}, 3^{rd} and 4^{th} & 5^{th}) as predictive factors. There were no significant differences in the FA composition of embryos sampled from both sides of the brooding chamber in 4 of the 5 surveyed females. However, all females exhibited significant differences in the FA profiles of embryos sampled from different pleopods. Similarly, for both saturated FA (SFA) and highly unsaturated FA (HUFA), FA profiles were not similar across the breeding chamber. Overall, FA reserves appeared to vary significantly within broods, which can ultimately be reflected on early larval survival. A potential cause for the within brood variation recorded in the FA profile of developing embryos include: 1) differential female investment during ovarian maturation, mainly due to variation in food quality/availability; 2) differential lipid catabolism during the incubation period of developing embryos, as a consequence of embryos position within the female's brooding chamber; or 3) differential female investment during ovarian maturation amplified by differential lipid catabolism during the incubation period.

2.2 Introduction

The Norway lobster, *Nephrops norvegicus* (Linnaeus, 1758), is a commercially important benthic decapod crustacean commonly found in the Northeastern (NE) Atlantic, from the coast of Iceland to Morocco and in the Mediterranean Sea (d'Udekem d'Acoz 1999). Its depth range extends from 15 to 800 m, although they are typically found on the NE Atlantic coast between 300 and 600 m depth (Tuck et al. 1997a). Several studies have already focused on different aspects of the biology and fisheries of *N. norvegicus*, but biochemical data related with the species reproductive cycle and embryonic development are still fairly recent and scarce (Rosa et al. 2003; Rosa and Nunes 2002; Rotllant et al. 2004; Tuck et al. 1997b). Mating occurs just after molting, shortly following the hatching from the previous year. Between 2 to 3 months later, an increase in ovary size is observed and during the following 4-5 months, vitellogenesis occurs (Rotllant et al. 2005). Spawned embryos are attached to the pleopods on the abdomen and incubated over an extensive period (up to 10 month in the NE Atlantic, Sardà 1995). In several decapods, and specifically in nephropid lobsters, ovarian maturation has been shown to have high energetic costs, embryonic development is lecithotrophic, i.e.: is entirely supported by egg yolk (Rosa and Nunes 2002).

In *N. norvegicus*, lipid profiles of developing ovaries seem to be more dependent on the ingestion of dietary lipids than on hepatopancreas reserves (Rosa and Nunes 2002). In this way, it is possible that female's feeding status during the extensive period over which vitellogenesis occurs can play a decisive role on hatching success and larval quality. Off the coast of mainland Portugal, *N. norvegicus* inhabits muddy substrate areas located between 400-800 m depth (dos Santos et al. 2007; Moita 2001). These deep-water habitats are characterized by a relative scarcity of available food resources (Labropoulou and Kostikas 1999), which may affect the nutritional state of females exhibiting developing ovaries and, consequently, potential energetic reserves available to fuel embryonic development. It is already known that lipids play a central role in embryonic metabolism, as they represent the most important energy source and form at least 60% of the total energy expenditure of developing crustacean embryos (Wehrtmann and Graeve 1998). In nephropid lobsters (e.g. *N. norvegicus* and

Homarus gammarus), lipid catabolism plays a crucial role during embryonic development, with up to70% of initial total lipids being depleted before hatching (Rosa et al. 2005; Rosa et al. 2003).

Among lipids, fatty acids (FA) are critical in embryonic development. Highly unsaturated FA (HUFA) with more than 20 C atoms are essential and therefore must be ingested from food as crustacean cannot, or only insufficiently, synthesized them *de novo* (Anger 2001). Long chain HUFA, such as the eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), play important roles in neuronal development, ion transport and temperature regulation. Because embryonic development is lecithotrophic and essential FA (EFA) cannot be synthesised *de novo*, these nutrients must be supplied by the female into the yolk reserves to ensure successful development. In contrast, saturated FA (SFA) can be synthesised from other FA and are oxidized to produce energy.

FA dynamics through embryonic development have been studied in detail for several decapod groups, namely caridean shrimp (e.g.: Graeve and Wehrtmann 2003), brachyuran crabs (e.g.: Torres et al. 2008) and nephropid lobsters, such as *Homarus gammarus* (Rosa et al. 2005) and *N. norvegicus* (Rosa et al. 2003). These studies allow us to understand the nutritional requirements exhibited by developing embryos along the incubation period. However, due to laboratorial constraints associated with the analytical techniques employed to perform FA analysis, egg samples of several females are commonly pooled together and inter-individual differences are not generally assessed. Additionally, to our knowledge, variability of FA profiles in the same egg mass has never been determined in any decapod crustacean. Lower oxygen availability has already been observed at the center of the condensed egg mass of brachyuran crabs (Fernandez et al. 2003). Potential deleterious effects resulting from this decrease in oxygen availability are commonly reduced through adaptations in the brooding behavior of ovigerous females (Baeza and Fernández 2002). Such brooding behaviors (e.g. abdominal flapping) have already been documented in ovigerous *N. norvegicus* (Eriksson et al. 2006). Embryonic metabolic rates commonly increase in reduced oxygen environment and yolk reserves provided by the female can be depleted at a faster rate (Brante et al. 2003). Although oxygen levels have so far never been

documented in *N. norvegicus*, hypoxia has already been shown to cause premature hatching, reduced embryonic survival and increased embryonic heart beat frequency in this species (Eriksson et al. 2006).

The objective of the present study was to investigate how homogeneous is the fatty acid profile of embryos in a single egg mass and test if embryos position along the female's brooding chamber (chamber side and pleopod to which embryos are attached) has any effect on their FA composition. Additionally, inter-individual variability in the FA profiles of *N. norvegicus* embryos was also assessed.

2.3 Material and Methods

2.3.1 Sampling

Five ovigerous *N. norvegicus* females (CL: 5.08 ± 0.38 cm), collected in January 2008 by a commercial fishing vessel using baited traps off the coast of Sagres (Southwestern Coast of mainland Portugal), at an approximate depth of 400 m, were selected for the present study. Across the brooding chamber, the embryos carried by each female displayed clearly visible eyes with 1/2 yolk consumed and were considered to be in development stage II (according to the scale proposed by Rosa et al. 2007). Embryos attached to each pleopod were carefully removed with a pair of forceps and stored in separate Eppendorfs. A preliminary analysis of eggs randomly sampled from each pleopod, indicated that there was no difference in egg size (1.27 ± 0.19 mm^{-3}). Additionally, a visual inspection revealed that, within a single female, eggs from different pleopods were at the same point in development.

Like most lobster, *N. norvegicus* ovaries are shaped like an elongated H and posses a right and left lobe connected by a small cross lobe (Rotllant et al. 2005). Because of the bilateral nature of the ovaries, left and right side pleopods were processed separately (not pooled). Due to the reduced number of embryos present in the 1st and 5th pair of pleopods, pooled samples of embryos were collected from the 1st and 2nd, as well as from the 4th and 5th, pair of pleopods, (see Fig. 1). In this way the following number of samples were collected: 2 sides of the brooding chamber (right and left) X 3 groups of

pleopods (1st & 2nd, 3rd and 4th & 5th) X 6 replicates X 5 females = 180 samples. Embryos were freeze-dried and stored at -32ºC for later biochemical analysis.

2.3.2 Fatty acid analysis

FA extraction and preparation of methyl esters were carried out according to Lepage and Roy (1986) modified by Cohen et al. (1988). Freeze-dried samples (100 mg) were transmethylated with 5 ml of methanol/acetyl chloride (95:5 v/v). The mixture was sealed in a light-protected Teflon-lined vial under nitrogen atmosphere and heated at 80°C for 1 h. The vial contents were then cooled, diluted with 1 ml water, and extracted with 2 ml of n-heptane. The heptane layer was dried over Na_2SO_4, evaporated to dryness under a nitrogen atmosphere and redissolved in heptane, which contained the methyl esters. The methyl esters were then analyzed by gas-liquid chromatography, on a VARIAN (Palo Alto, USA) 3800 gas-liquid chromatograph (USA), equipped with a flame ionization detector. Separation was carried out on a 0.32 mm × 30 m fused silica capillary column (film 0.32 μm) Supelcowax 10 (SUPELCO, Bellafonte PA, USA) with helium as carrier gas at a flow rate of 1.3 ml min^{-1}. The column temperature was programmed at an initial temperature of 200°C for 10 min, then increased at 4°C min^{-1} to 240°C and held there for 16 min. Injector and detector temperatures were 250 and 280°C, respectively, and split ratio was 1:100. Peak identification was carried out using known standards (GC 462, Nu-Chek-Prep, Elysian, USA). Peak areas were determined using the Varian software and the FA 21:0 was used as an internal standard. The detection limit for the FAs analyzed was 0.01 μg mg^{-1} dry weight.

2.3.3 Statistical analysis

In order to visualize inter-individual differences in FA profiles, a Principal Coordinate Analysis (PCO) was performed, representing differences between all replicate for each female along the first two axes. Multivariate statistical analyses were performed to detect intra-individual differences in: 1) FA composition of *N. norvegicus* embryos, but only considering those representing more than 5% of total FA; 2) SFA composition; and 3) HUFA composition. We decided to specifically test the differences in SFA and HUFA

composition due to the role that SFA catabolism plays to fuel the energetic demands of embryonic development and because several HUFAs are already known to be essential FAs for decapods (*e.g.* eicosapentaenoic acid - EPA, docosahexaenoic acid - DHA) (Anger 2001). Prior to statistical analysis, all data were square root transformed, in order to reduce the contribution of highly and less abundant FA. A resemblance matrix was computed using Bray-Curtis similarity.

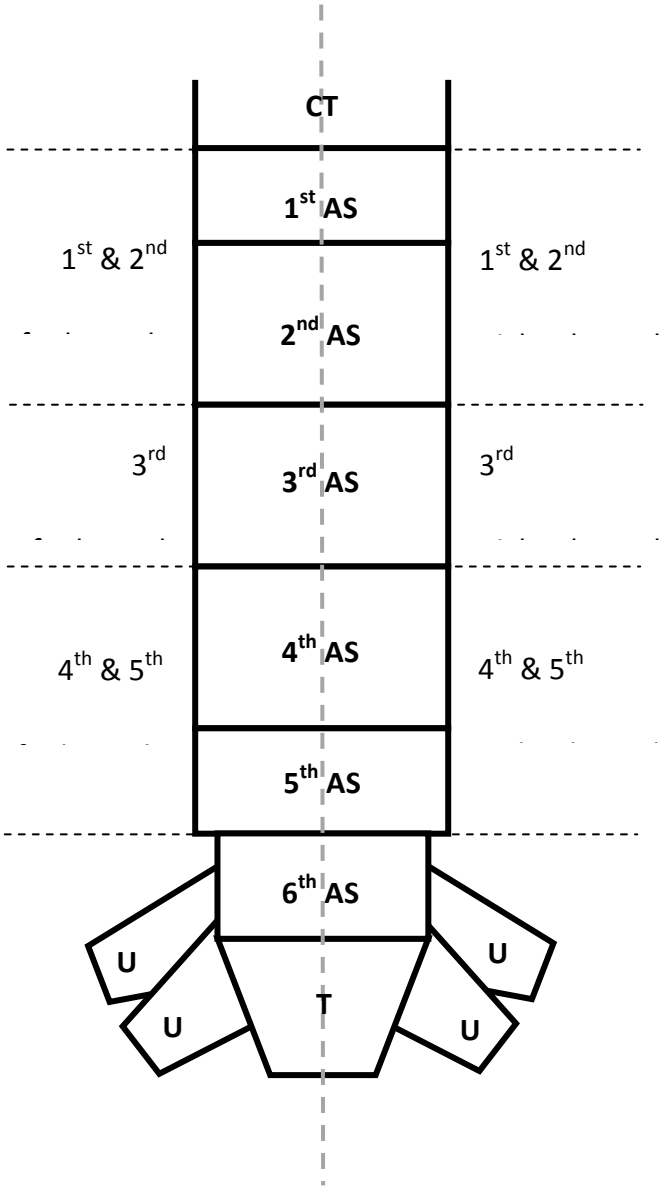

Figure 2.1: Schematic representation of *Nephrops norvegicus* female's abdominal ventral view illustrating the six distinct regions of the brooding chamber where developing embryos were sampled for fatty acid analysis (the egg mass attached to the 1st and 2nd pleopods was pooled, as well as that from the 4th and 5th pleopods). CT – cephalothorax, AS – abdominal segment, T – telson and U – uropod.

Factor pleopod to which embryos are attached was tested based on the anteroposterior "migration" taking place during egg spawning in *N. norvegicus* (the first embryos spawned migrate towards the posterior end of the brooding chamber) (unpublished data). Factor brooding chamber side was tested due to the elongated H like shape displayed by *N. norvegicus* ovaries, with their right and left lobes connected by a small cross-lobe at the level of the heart (Rotllant et al. 2005). The effect of factor pleopod (3 levels: 1^{st} & 2^{nd}, 3^{rd} and 4^{th} & 5^{th}) and factor brooding chamber side (2 levels: left and right) on FA composition were determined separately for each female using an analysis of similarities (ANOSIM).

ANOSIM calculates a global R statistic that assesses the differences in variability between groups, as compared to within groups, and checks for the significance of R using permutation tests (Clarke and Gorley 2006). Differences in FA profiles recorded for pleopod and side were explored using the similarity percentages routine (SIMPER). SIMPER decomposes average Bray-Curtis dissimilarities between all pairs of samples, into percentage contributions from each FA, listing FAs in decreasing order of their contribution for the dissimilarity (Clarke and Gorley 2006). All statistical tests were performed with Primer 6.1 with PERMANOVA add-on (Primer-E Ltd, Plymouth, UK). All tests used 9999 permutations.

2.4 Results

The result of the PCO analysis indicated that, despite high within-brood variability, there were difference in FA profiles, especially between female 2, 3 and 5 (Figure 2). Within each brooding chamber, a comparison of the size of the embryo, eye and the yolk sack that embryos of different pleopods were of similar and therefore at the same point in development. The average FA composition of *Nephrops norvegicus* embryos of each sampled female is shown in Table 1. In all analyzed females (n=5), the quantitatively most abundant FAs were 22:6*n*-3 (DHA, representing 20.8 ± 3.9% (average ± standard error) of total FA), 18:1*n*-9 (oleic acid, 19.5 ± 2.0%), 16:0 (palmitic acid, 15.2 ± 3.4%), 20:5*n*-3 (EPA, 10.2 ± 1.4%), 16:1*n*-7 (palmitoleic acid, 8.9 ± 1.6%), and 18:1*n*-7 (cis-vaccenic acid, 5.7 ±1.3%).

Table 2.1: *Nephrops norvegicus*: Average embryonic FA composition (μg/mg DW ± SD) of eggs at stage II of embryonic development for each of the five tested female.

Fally Acid (μg/mg DW)	Female 1 Mean ± SD	Female 2 Mean ± SD	Female 3 Mean ± SD	Female 4 Mean ± SD	Female 5 Mean ± SD
14:0	1.39 ± 0.93	1.80 ± 0.86	2.40 ± 0.62	1.47 ± 0.82	0.54 ± 0.22
16:0	14.61 ± 6.11	16.26 ± 5.94	22.91 ± 4.23	10.81 ± 3.87	8.90 ± 2.20
17:0	0.15 ± 0.24	0.51 ± 0.43	1.09 ± 0.25	0.52 ± 0.20	0.72 ± 0.49
18:0	3.20 ± 0.81	4.24 ± 1.85	5.65 ± 1.06	3.93 ± 1.17	3.68 ± 0.68
20:0	0.09 ± 0.17	0.18 ± 0.28	0.33 ± 0.33	0.50 ± 0.32	0.51 ± 0.18
21:0	2.84 ± 0.71	2.33 ± 0.48	3.49 ± 0.65	4.56 ± 0.58	2.85 ± 0.27
22:0	0.12 ± 0.73	0.20 ± 0.37	0.21 ± 0.29	0.35 ± 0.26	0.36 ± 0.15
24:0	0.29 ± 0.42	0.29 ± 0.69	0.23 ± 0.35	0.56 ± 0.32	0.36 ± 0.14
ΣSFA	**22.69 ± 10.11**	**25.80 ± 10.89**	**36.34 ± 7.78**	**22.71 ± 7.55**	**17.91 ± 4.34**
16:1 n-7	10.00 ± 4.02	10.94 ± 3.96	11.74 ± 2.26	6.39 ± 2.37	4.57 ± 1.06
18:1 n-9	18.72 ± 6.02	18.13 ± 5.13	26.07 ± 3.15	14.11 ± 2.88	16.15 ± 5.31
18:1 n-7	6.31 ± 2.85	5.36 ± 2.00	7.44 ± 1.58	3.87 ± 0.85	4.16 ± 0.69
22:1 n-9	0.68 ± 0.54	0.12 ± 0.19	0.20 ± 0.23	0.63 ± 0.26	0.32 ± 0.13
ΣMUFA	**35.72 ± 13.43**	**34.55 ± 11.28**	**45.45 ± 7.22**	**24.99 ± 6.36**	**25.19 ± 7.19**
18:2 n-6	0.71 ± 0.35	0.86 ± 0.52	2.48 ± 0.28	1.24 ± 0.31	1.79 ± 1.83
18:3 n-6	0.00 ± 0.03	0.08 ± 0.22	0.18 ± 0.23	0.19 ± 0.24	0.32 ± 0.12
18:3 n-3	0.16 ± 0.23	0.50 ± 1.01	0.83 ± 0.27	0.49 ± 0.16	0.51 ± 0.33
20:2 n-6	0.42 ± 0.35	0.34 ± 0.38	1.37 ± 0.10	0.90 ± 0.09	0.91 ± 0.11
20:4 n-6	2.87 ± 0.94	2.19 ± 0.62	3.02 ± 0.41	3.05 ± 0.42	2.37 ± 0.27
20:3 n-6	0.07 ± 0.17	0.11 ± 0.42	0.27 ± 0.31	0.25 ± 0.21	0.28 ± 0.17
20:5 n-3	8.73 ± 1.16	7.15 ± 1.66	13.00 ± 1.19	10.96 ± 1.64	7.97 ± 0.69
22:4 n-6	0.27 ± 0.30	0.07 ± 0.17	0.07 ± 0.13	0.22 ± 0.18	0.29 ± 0.14
22:5 n-3	1.41 ± 0.58	1.87 ± 0.50	1.98 ± 0.18	2.33 ± 0.25	1.60 ± 0.28
22:6 n-3	20.84 ± 3.09	15.67 ± 2.07	22.60 ± 1.38	19.89 ± 1.85	15.83 ± 1.74
ΣPUFA	**35.47 ± 7.21**	**28.84 ± 7.58**	**45.80 ± 4.48**	**39.53 ± 5.36**	**31.88 ± 5.67**
ΣHUFA	**34.10 ± 6.08**	**26.97 ± 5.03**	**40.67 ± 3.29**	**36.45 ± 4.34**	**28.06 ± 3.12**
ΣUFA	**71.19 ± 20.64**	**63.39 ± 18.86**	**91.25 ± 11.70**	**64.52 ± 11.72**	**57.07 ± 12.85**

Pleopod and side affected embryonic FA profiles differently, according to female or the group of FA tested (Fig. 3 and 4, Table 2). When only the FA representing at least 5% of total FA composition were considered in the analysis, there was a significant effect of factor pleopod in all females (ANOSIM: $R > 0.171$; $p < 0.008$). Considering factor side, there was no significant effect on females 1 to 4 (ANOSIM: $R < 0.074$; $p > 0.098$) and only on female 5 was recorded a significant effect of brooding chamber side on the FA profiles of developing embryos (ANOSIM: $R = 0.171$; $p = 0.018$). With the exception of female 2, the embryos located in 1^{st} & 2^{nd} and 3^{rd} pleopods of all females exhibited similar FA profiles. However, the FA profiles of these embryos differed from those recorded on embryos located in the 4^{th} & 5^{th} pleopod (Fig. 3). In general, the FAs responsible for the differences detected among samples were, in decreasing order,

16:0, 16:1*n*-7, 18:0, 18:1*n*-7 and 18:1*n*-9 (with the contribution of each FA for those differences being > 10%; SIMPER).

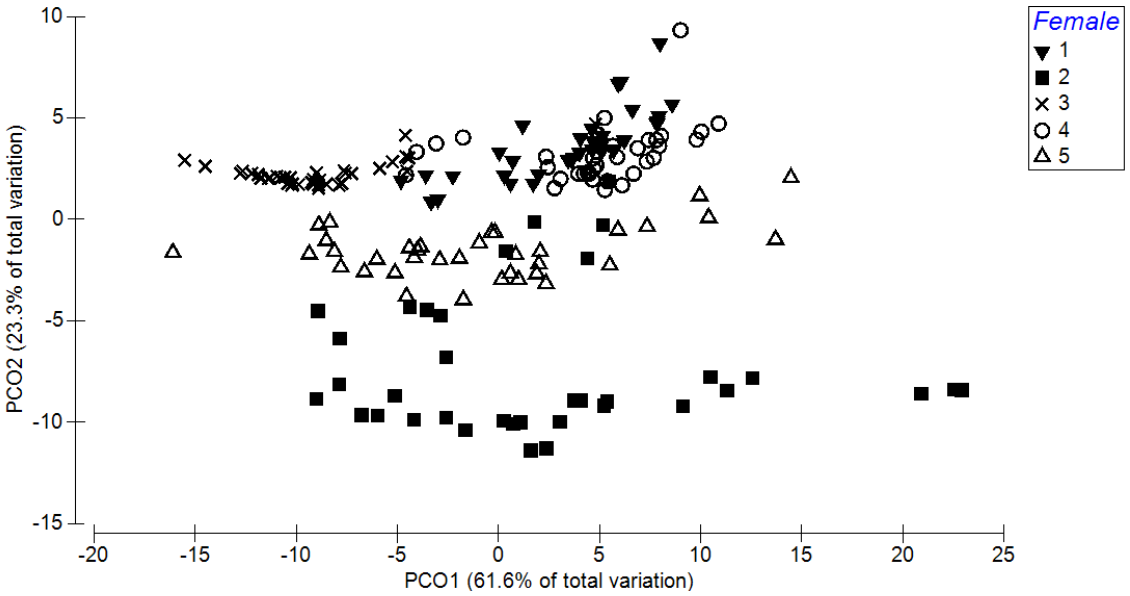

Figure 2.2: *Nephrops norvegicus*: Principal Coordinate Analysis (PCO) comparing the fatty acid (FA) profiles of the embryos of five different females. All embryos were at stage II of development.

Table 2.2: *Nephrops norvegicus*: P values resulting from the ANOSIM analysis comparing the fatty acid (FA) profiles between pleopods and sides for each female. The tests were performed with the FA present at an abundance over 5% of total FA (FA>5%), the Saturated FA (SFA) and the highly unsaturated FA (HUFA). The "ns" indicate that the difference is not significant (α=0.05).

Female	Factor	FA> 5%	SFA	HUFA
1	Pleopod	0.0001	0.0001	ns
	Side	ns	ns	ns
2	Pleopod	0.008	0.005	0.0001
	Side	ns	0.025	0.011
3	Pleopod	0.009	0.0002	0.013
	Side	ns	0.017	ns
4	Pleopod	0.0002	ns	0.002
	Side	ns	ns	0.002
5	Pleopod	0.006	0.0001	0.009
	Side	0.018	0.0001	0.038

Considering the SFA profile alone, there was a significant difference in the FA composition between different pleopods in all females (with the exception of female 4) (ANOSIM: R > 0.192; p < 0.009) (Fig. 4). Females 2, 3 and 5 also exhibited a significant effect of side in the FA composition of their embryos (ANOSIM: R > 0.169; p < 0.025). None of the sampled females displayed a defined trend in embryo SFA content, as it increased or decreased along the anterior-posterior axis of the brooding

chamber. Only female 4 displayed a uniform SFA composition of developing embryos along the whole brooding chamber (ANOSIM: $R < 0.067$; $p > 0.094$). In general, the SFAs responsible for the differences detected among samples were, in decreasing order, 14:0, 16:0, and 18:0 (with the contribution of each one for those differences being > 10%; SIMPER).

The analysis of embryonic HUFA indicated that there was a significant effect of factor pleopod (ANOSIM: $R > 0.211.$; $p < 0.002$) and side (ANOSIM: $R > 0.204$; $p < 0.011$) in females 2, 4 and 5 (Fig. 5). Female 3 displayed significant differences in HUFA composition of embryos located in different pleopods (ANOSIM: $R = 0.16$; $p = 0.013$), but not on different sides of the brooding chamber (ANOSIM: $R = 0.102$; $p = 0.063$). Finally, female 1 displayed a uniform HUFA composition in embryos located along the brooding chamber (ANOSIM: $R < 0.06$; $p > 0.163$). In general, the HUFAs responsible for the differences detected among samples were, in decreasing order, 20:4*n*-6 (arachidonic acid, ARA), 20:5*n*-3 (EPA), 22:5*n*-3, and 22:6*n*-3 (DHA) (with the contribution of each one for those differences being > 10%; SIMPER).

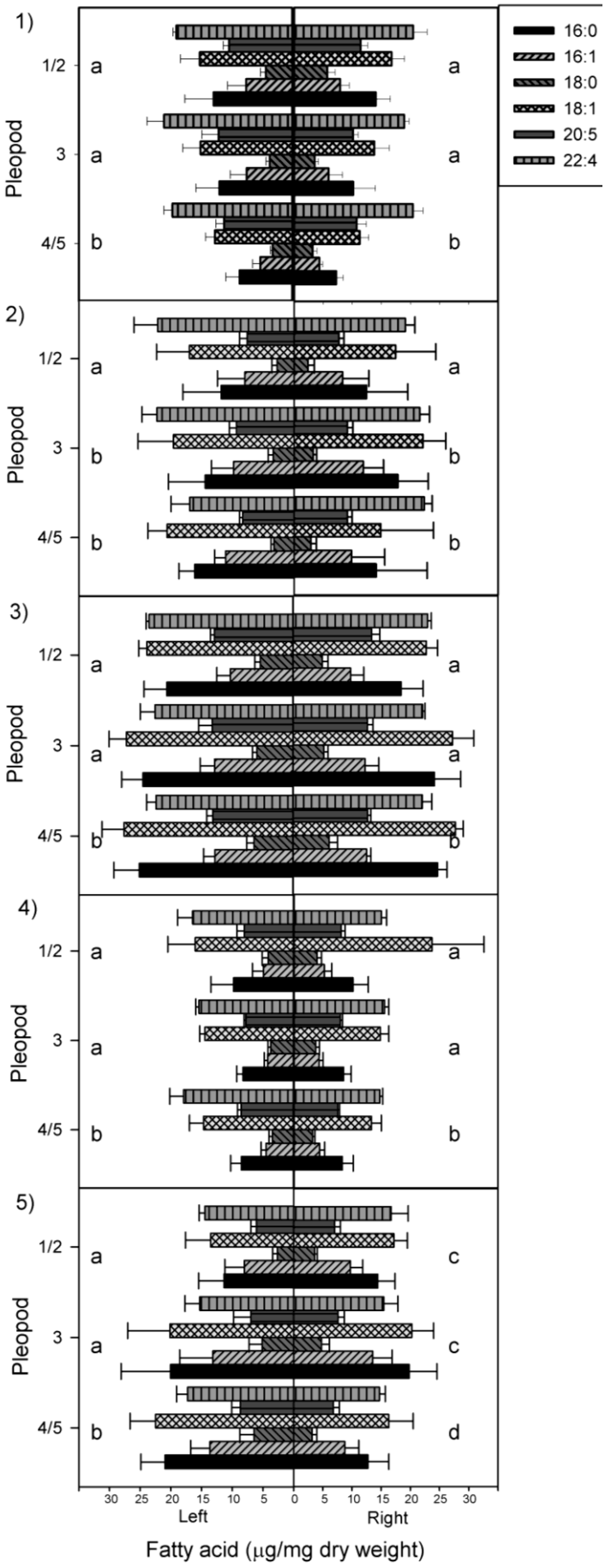

Figure 2.3: *Nephrops norvegicus*: Embryonic fatty acids (FA) (μg/mg dry weight) (± standard error) present at an abundance over 5% of total FA across the brooding chamber of 5 females (numbers 1 to 5), plotted according to side (x-axis) and pleopods (y-axis). Pleopods 1 and 2 as well as 4 and 5, were grouped together for the analysis. Different letters represent embryos with significantly different FA composition (α=0.05). For clarity only the 6 most abundant FA are depicted in the figure even though 18:1 *n-7*, 18:2 *n-6* and 18:3 *n-7* were included in the statistical analysis.

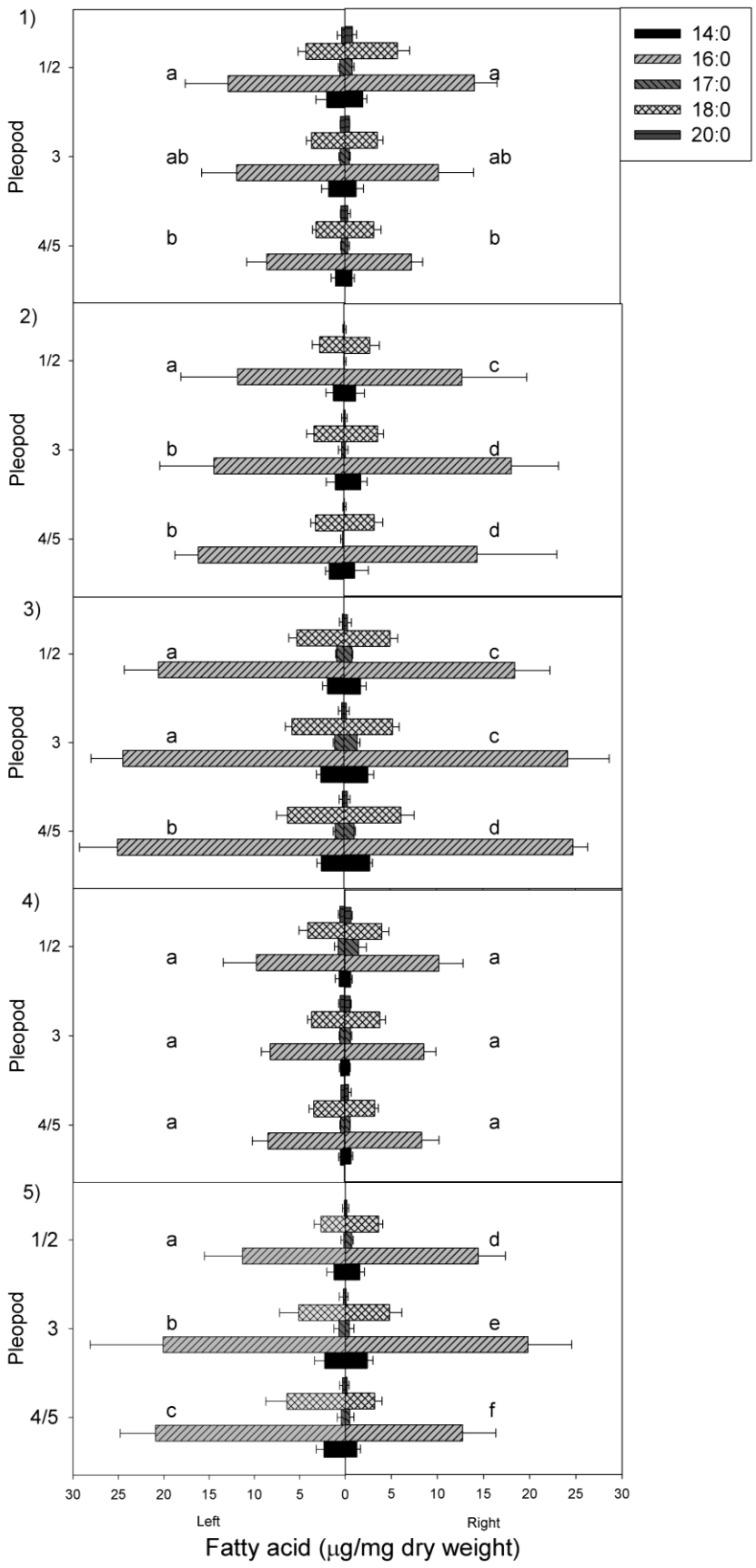

Figure 2.4: *Nephrops norvegicus*: Embryonic saturated fatty acids (SFA) (μg/mg dry weight) (± standard error) across the brooding chamber of 5 females (numbers 1 to 5), plotted according to side (x-axis) and pleopods (y-axis). Pleopods 1 and 2 as well as 4 and 5, were grouped together for the analysis. Different letters represent embryos with significantly different FA composition (α=0.05). For clarity only the 5 most abundant SFA are depicted in the figure even though 22:0 and 24:0 were included in the statistical analysis.

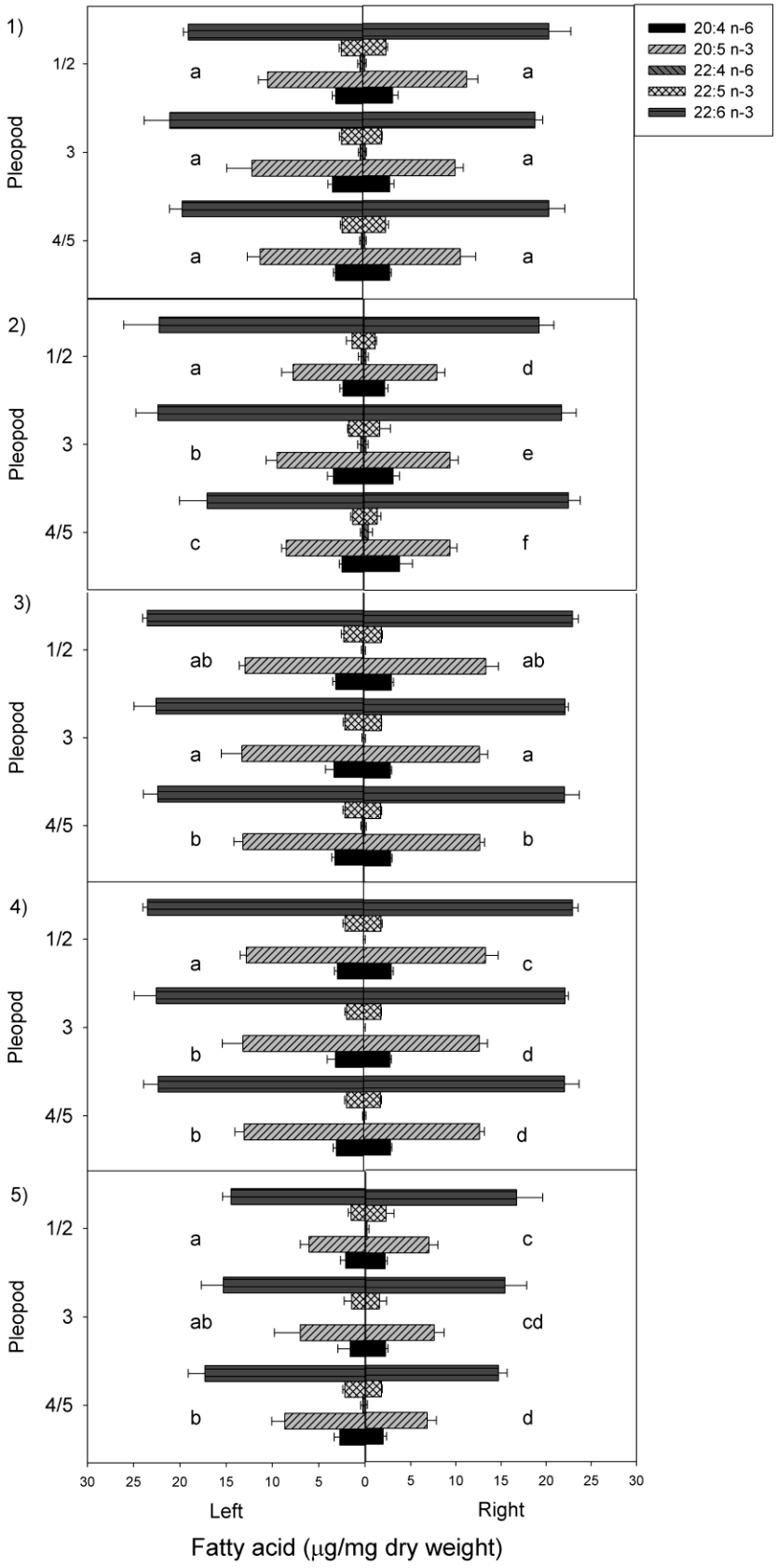

Figure 2.5: *Nephrops norvegicus*: Embryonic highly unsaturated fatty acids (HUFA) (μg/mg dry weight) (± standard error) across the brooding chamber of 5 females (numbers 1 to 5), plotted according to side (x-axis) and pleopods (y-axis). Pleopods 1 and 2 as well as 4 and 5, were grouped together for the analysis. Different letters represent embryos with significantly different FA composition (α=0.05).

2.5 Discussion

A strong inter-individual and within brood variability was recorded in the FA profiles displayed by developing embryos of *N. norvegicus*. The FA profile of developing embryos is known to change significantly over time and stage II of embryonic development may last a couple of months (Rosa et al. 2003). In this way, as egg extrusion may have not occurred simultaneously in all sampled specimens, different females may have carried early or late developing stage II embryos at sampling. This aspect alone may explain the inter-individual variability recorded in embryonic FA profiles.

Overall embryonic FA composition was somewhat different from that previously recorded for *N. norvegicus* off the coast of Portugal (Rosa et al. 2003). The order of the 5 most abundant FA in stage II of embryonic development was different between the two studies. Additionally, the average content of individual FA (per mg of dry weight) was around half the value reported by Rosa et al. (2003). This difference in the absolute level of FA is also not surprising, as studies on recently hatched *N. norvegicus* larvae have shown that their biochemical composition may vary significantly among locations (Álvarez-Fernández et al. 2009; Rotllant et al. 2004). However, the sampling sites of this study and that by Rosa et al. (2003) are only separated by about 200 km. Shifts in food resources influencing growth have been shown to affect ovarian lipid composition (Tuck et al. 1997b). Therefore, it is possible that the difference recorded in the FA composition of developing embryos may rather be explained by inter-annual differences in female dietary regimes, than by spatial variability.

Only two HUFA, EPA and DHA, were recorded in comparable levels as in the previous study by Rosa et al. (2003). Both FA are known to play an important developmental and structural role (Bell and Dick 1990; Fox et al. 1994; Stanley-Samuelson 1987). Since embryonic development in *N. norvegicus* is lecithotrophic, and HUFA cannot be synthesized *de novo*, these essential FAs must be derived from the diet during gametogenesis (Rosa et al. 2007). Some mechanisms may exist to maintain the levels of these FA relatively stable both at oviposition, and later through embryonic development, even if the FA profile of the food ingested by the female is variable.

Indeed, in the spiny lobster *Jasus edwardsii*, lipid, and more specifically HUFA composition of both ovaries and newly hatched larvae was not affected by the lipid content of the diet (Smith et al. 2004). Within brood variability recorded for the SFA profiles of developing embryos between this study and previous ones (Rosa et al. 2007) may ultimately explain variability recorded in early larval survival. In fact, early larval performance was found to be associated with high levels of SFA, as energy is released more efficiently through the oxidation of these FA (Turner et al. 2003). Nevertheless, during embryonic development, SFA should be depleted first as they are catabolized as an energy source while essential fatty acid should be retained for growth and development. Off the coast of mainland Portugal, *N. norvegicus* larvae hatch between 400 and 800 m (dos Santos et al. 2007; Moita 2001), and therefore in an oligotrophic environment. Newly hatched larvae then undergo a migration towards the food rich photic zone near surface to forage for suitable prey (dos Santos and Peliz 2005). In the presence of food, zoea I will readily ingest prey immediately after hatching (Pochelon et al. 2009). However, laboratorial studies have also indicated that newly hatched *N. norvegicus* larvae can endure periods of complete food deprivation (> 24 h) by catabolizing embryonic reserves to fulfill their energetic needs (unpublished data). Given that *N. norvegicus* larvae are planktotrophic, qualitative and quantitative differences in embryonic reserves, including lipid, will condition their ability to cope with periods of starvation following hatching. This has already been documented for many decapod species, such as the spiny lobster *Jasus edwardsii* (Johnston et al. 2004; Ritar et al. 2003a), the anomuran crabs *Lithodes santolla* and *Paralomis granulosa* (Kattner et al. 2003) and several penaeid shrimp (D'Abramo 1989).

As the embryos analyzed were at stage II of embryonic development, the within brood variability recorded for their FA profiles can either result from: 1) differential female investment during ovarian maturation, mainly due to variation in food quality/availability; 2) differential lipid catabolism during the incubation period of developing embryos, as a consequence of embryos position within the female's brooding chamber; or 3) differential female investment during ovarian maturation amplified by differential lipid catabolism during the incubation period. As stated above,

lipid reserves available for embryonic development can only be provided by the female prior to oviposition. The availability of FA during ovarian maturation will therefore be dependent on the quantity and/or quality of dietary items ingested by the female. Previous studies in lobsters (Smith et al. 2004), and shrimp (Cahu et al. 1995; Calado et al. 2010a; Racotta et al. 2003) indicated that food supplied to females significantly affect the FA profiles displayed by produced embryos. Nonetheless, as ovarian maturation is a continuous process in *N. norvegicus* (Rotllant et al. 2005), it's not likely that the differences recorded in the FA profiles of embryos may result from uneven nutrient allocation during vitellogenesis.

Alternatively, differences in environmental conditions within the brooding chamber may promote a shift in the FA consumption dynamics during the incubation period. It has already been described for brachyuran crabs that within the dense embryo mass oxygen concentration can be variable (Fernandez et al. 2003). Contrasting oxygen levels can cause variations in embryonic metabolic rates (Brante et al. 2003), which will therefore increase energy demand and SFA oxidation. This scenario will cause a differential catabolism of embryonic reserves and eventually lead to asynchronous larval hatching (Eriksson et al. 2006; Fernandez et al. 2003). Indeed, *N. norvegicus* (personal observation) and *Homarus americanus* (Pandian 1970), have been recorded to hatch their larvae over several consecutive nights. These episodes were often considered as a "laboratorial artifact", but the present data evidencing within brood variation in embryonic FA profiles supports the potential occurrence of asynchronous embryonic development in the wild, which may ultimately promote asynchronous larval hatching. Ultimately, early larval survival will also be affected. Since embryonic development is lecithotrophic and food might not be readily available immediately following birth, it can be assumed that the only energy available to the larvae come from the yolk reserves. The larvae that had lower energetic embryonic input may not be able to successfully reach the following larval stage, even though short term survival to suboptimal food conditions can still be possible (e.g.: Calado et al. 2010b; Gebauer et al. 1999; Giménez and Anger 2003, 2005; Ritar et al. 2003b; Smith et al. 2004).

As the present work was performed on stage II embryos, it is impossible to determine whether it is maternal investment or embryonic catabolism that is responsible for the variability recorded in embryos FA profiles. A continuous monitoring of lipid catabolism displayed by developing embryos located in different regions within the same brooding chamber, from oviposition to hatching, will certainly help researchers to clarify the biochemical variability recorded for *N. norvegicus* embryos (e.g. by monitoring the FA profile of embryos along the brooding chamber immediately after spawning, it will be possible to determine if maternal investment in yolk reserves is indeed uniform). Additionally, by monitoring oxygen concentration along the brooding chamber through embryonic development, researchers can better understand the potential sources of variable egg yolk catabolism within the same egg mass. After performing such studies it will be possible to determine if and how pre-hatching latent effects are responsible for the differences recorded in nephropid larvae hatching synchronism and survival.

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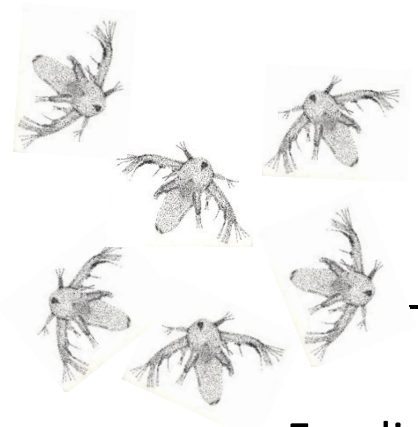

Chapter 3

Feeding ability of early zoeal stages of the Norway lobster *Nephrops norvegicus* (L.)

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Chapter 3

Feeding ability of early zoeal stages of the Norway lobster *Nephrops norvegicus* (L.)

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Key words - Feeding ability, *Nephrops norvegicus*, Zoea

3.1 Abstract

The wide geographical distribution of the Norway lobster, *Nephrops norvegicus*, results in a delay, with latitudinal decrease, in the larval season from spring to winter. Newly hatched larvae of the species may therefore be exposed to suboptimal levels/types of prey availability and face intermittent periods of starvation at low latitudes. This work investigated the feeding response of the first two zoeal stages of *N. norvegicus* under variable prey densities, prey types, feeding histories and photoperiods. Both zoeae (Z) I and II increased the number of consumed prey with increasing food levels. ZI preferred *Artemia* sp. nauplii over larger metanauplii, while in ZII, higher ingestion was only observed for metanauplii at higher food concentrations. The number of prey ingested by larvae previously starved or under low food conditions was always higher than that of larvae exposed to high food levels. These findings seem to indicate that larvae may maximize prey ingestion in the presence of plankton patches with higher food abundance and minimize the deleterious effects induced by previous periods of intermittent starvation or unsuitable prey densities/types. Extreme photoperiods (24 and 0h of light) did not improve larval feeding ability and are not a suitable option for larviculture.

3.2 Introduction

The Norway lobster, *Nephrops norvegicus* (Linnaeus, 1758), is a commercially important benthic decapod crustacean commonly found in the Northeastern Atlantic waters from the Coast of Iceland to Morocco and in the Mediterranean Sea (d'Udekem d'Acoz 1999). Its depth range extends from 15 to 800 m, although they are typically found on the NE Atlantic shelf between 300 and 600 m depth (Tuck et al. 1997a) and 200 and 800 m in the Mediterranean (Maynou and Sardà 1997). In addition to differences in the depth range, the reproduction period of this species also varies latitudinally, with average embryo incubation being 10 months in the Northeastern Atlantic but only 6 months in the Mediterranean (Sardà 1995). These differences in embryo incubation duration are known to affect the hatching period of *N. norvegicus*

larvae, with hatching occurring by early spring in the Northeastern Atlantic and at the end of winter in the Mediterranean (Rotlland et al. 2004). Off the Coast of Portugal, adults are encountered at depth ranging from 400 to 800 m and the hatching period extends from December until April with however some regional differences, larval release peaking earlier in the south than the north. Under these scenarios, early larval development at the end of winter will occur in a nutritionally poor environment, since chlorophyll *a* peak only occurs during spring (Moita 2001; Santos et al. 2007). Late larval release will overlap with the beginning of thermal stratification and spring bloom (Santos et al. 2007).

Regardless of geographical and seasonal differences in nutritional conditions, it is widely accepted that planktonic larvae are naturally subjected to intermittent periods of starvation and/or suboptimal prey availability due to the natural patchiness of plankton distribution in the oceanic environment (Andersen and Nielsen 2002; Folt and Burns 1999; Pinel-Alloul 1995). Studies addressing larval feeding in clawed lobsters have highlighted how early larval feeding greatly affects their development, since these organisms may not recover from nutritional stress if suitable food is not available shortly after hatching (Anger et al. 1985; Rotlland et al. 2001). Newly hatched *N. norvegicus* larvae from the Mediterranean are larger in size and richer in lipids and proteins than those from the Irish sea, which emphasize the major role that these features may play for larval survival in oligotrophic environments (Rotlland et al. 2004).

Larvae submitted to low food conditions use several feeding strategies, and may either morphologically (Fenaux et al. 1994; Strathmann et al. 1993) or behaviorally (McConaugha 2002) enhance their overall feeding efficiency. Planktonic larvae of many benthic species appear to have evolved a certain feeding plasticity, in order to be able to feed over a broad range of prey sizes, types and abundances (Hinz et al. 2001; McConaugha 2002; Perez and Sulkin 2005; Strathmann and Bone 1997). By feeding on smaller and/or suboptimal food items, developing larvae may minimize the negative effects of low food density and avoid starvation. In fact, this feeding plasticity had already been previously documented in homarid lobsters, with the last zoeal stage of *Homarus americanus* being recorded to successfully perform suspension feeding (in

contrast to the most commonly recorded raptorial feeding behavior) (Barshaw and Bryant-Rich 1988).

Despite the number of studies addressing *N. norvegicus* larval biology (Briggs et al. 2002; Dickey-Collas et al. 2000a; dos Santos and Peliz 2005; Rotlland et al. 2004) and aquaculture potential (Anger and Puschel 1986; Figueiredo and Vilela 1972; Morais et al. 2001; Rosa et al. 2003; Rotlland et al. 2001), little is still known about the feeding ability of early zoeal stages.

Our objective was to assess the feeding ability of *N. norvegicus* zoea (Z) I and II under variable feeding scenarios and to identify the existence of feeding plasticity in these early zoeal stages. In this way, the feeding response of *N. norvegicus* ZI and ZII under variable prey densities, prey types and experiencing previous feeding histories was investigated. Additionally, due to the current interest in the larviculture of *N. norvegicus*, mainly for restocking purposes, the effect of extreme photoperiods (24 and 0 hours of light) on prey consumption for both zoeal stages was also evaluated.

3.3 Material and Methods

3.3.1 Larval production and selection

Five *N. norvegicus* ovigerous females were collected between February and April 2008 using baited traps near Peniche (West Coast of Portugal), at an approximate depth of 400 m, and kept in the laboratory until their larvae hatched. Newly hatched larvae were either selected for the experiments described bellow or reared to the second zoeal stage using a recirculating larviculture system employing 20 l cylindrico-spherical culture tanks (see Calado 2008 for further details). Larvae were fed daily with newly hatched *Artemia* sp. nauplii (San Francisco Bay Brand Inc., Newark, CA, USA) supplied at a density of 5 nauplii ml^{-1}, with *Artemia* sp. cysts being incubated according to the procedures described by Sorgeloos et al. (1998). During the culture period, *N. norvegicus* larvae were maintained in constant darkness at 15±1ºC in a temperature-controlled culture room. Natural seawater at a salinity of 35 was 1 µm filtered, passed through activated carbon and UV irradiated before being used in the

larviculture system. Larvae were only used in the experimental trials 24 h after hatching or molting to the second zoeal stage, in order to guarantee that tested specimens already displayed larval appendages involved in prey capture, manipulation and ingestion that were fully functional. Additionally, only those specimens displaying strong phototactic responses were selected for the experimental trials, since this behavior is commonly employed in crustacean aquaculture to evaluate larval quality (see Treece and Fox 1993). In all experimental trials, larvae at both zoeal stages were placed individually in 40 ml plastic beakers with filtered seawater at a salinity of 35 in a climatized chamber at 15±1ºC. Larvae recorded as dead or moribund (laying motionless in the bottom of the plastic beaker for prolonged periods but still beating the exopods of their pereiopods) at the end of experimental trials were discarded and those replicates repeated. To ensure a homogenized distribution of prey in the water column during the experimental trials, minimizing the positive phototactic behavior of larvae and dietary prey, each plastic beaker was gently aerated at a rate of ca. one air bubble per second.

3.3.2 Effect of prey densities and photoperiods

The feeding ability of *N. norvegicus* larvae was evaluated by providing to both ZI and ZII four different prey densities (0.5, 1, 3, and 5 *Artemia* sp. nauplii ml^{-1}) under three distinct photoperiods (24, 12 and 0 h of light). After 24 h, the number of live prey remaining in the plastic beaker was counted under a binocular stereomicroscope, with damaged *Artemia* sp. nauplii (e.g. missing one or both antenna or their abdomen) being considered as ingested. Five replicates were used for each treatment for a total of 60 specimens (4 densities × 3 photoperiods × 5 replicates = 60) per each larval stage. The variable used to assess the feeding ability of both ZI and ZII was the total number of larval prey ingested after 24 h.

3.3.3 Effect of prey types and prey densities

The combined effect of four different prey types (*Artemia* sp. nauplii (Naup, average size 450 ± 10 µm), *Artemia* sp. metanauplii (Meta, average size 590 ± 5 µm), *Artemia* sp. metanauplii enriched with the commercial product Algamac™ 2000 produced by Aquafauna, Biomarine Inc., Hawthorne, CA, USA (MetaAlga, average size 592 ±7 µm)

and enriched with the spray dried microalgae *Spirulina* (MetaSpi, average size 591 ± 6 µm)) and four prey densities (0.5, 1, 3, and 5 prey ml^{-1}) on the feeding ability of the first and second zoeal stages of *N. norvegicus* was evaluated. The enrichments were conducted according to the protocols described by Morais *et al.* (2001) during 16h in 1 l beakers, with strong aeration and a maximum density of 50 nauplii.ml-1; 0.1g of product was used in 1 l of seawater. All trials were performed under a 12 h light :12 h dark photoperiod. After 24 h the number of live dietary prey in each beaker was counted using the methods and criteria described above. Five replicates were used for each treatment for a total of 80 specimens (4 prey types × 4 densities × 5 replicates = 80) per each larval stage. The variable used to assess the feeding ability of both ZI and ZII under the experimental conditions was the total number of larval prey ingested after 24 h.

3.3.4 Effect of previous feeding histories

In order to investigate the feeding ability of *N. norvegicus* ZI and II previously exposed to different feeding histories, larvae were acclimated for 12 h to three different feeding scenarios: starvation, low prey density (0.5 dietary prey ml-1), and high prey density (5 dietary prey ml-1). After the 12 h acclimation period, larvae from each feeding scenario were placed either under low or high prey densities (0.5 and 5 dietary prey ml-1, respectively) for an additional 12 h. The dietary prey provided to ZI and ZII were newly hatched *Artemia* sp. nauplii and *Artemia* sp. metanauplii enriched with Algamac™ 2000, respectively. The two stages were not fed with the same prey item since each stage displayed a different preference in the experiment “effect of prey types and prey densities”. ZI ate more nauplii and ZII more MetaAlga. All trials were performed under a 12 h light :12 h dark photoperiod. At the end of the experimental period, the number of live dietary prey was counted according to the criteria previously described. Five replicates were used for each treatment for a total of 30 specimens (6 feeding histories × 5 replicates = 30) per each larval stage. The variable used to assess the feeding ability of both zoeal stages was the total number of larval prey ingested in the 12 hours following the end of the acclimation period.

3.3.5 Statistical analysis

The feeding ability of the two first zoeal stages of *N. norvegicus* under the first two experimental conditions was compared using multi-way factorial analysis of variance ANOVA. The factors tested for each ANOVA were: prey densities (4 levels), photoperiods (3 levels), and zoeal stage (2 levels) for the experiment "Effect of prey densities and photoperiods"; prey types (4 levels), prey densities (4 levels), and zoeal stage (2 levels) for the experiment "Effect of prey types and prey densities". Finally, for the experiment "Effect of feeding histories" a one-way ANOVA (6 levels) was used separately for each zoeal stage. Statistical analyses were performed using the software STATISTICA 6.0 (manufactured by StatSoft Inc., USA). Prior to analysis, assumptions were verified and data transformed whenever necessary. Whenever significance was accepted, at $P < 0.05$, the Tukey multiple comparison test was used (Zar 1999). Subsequently, average prey consumption will always be reported with its standard deviation (mean ± SD).

3.4 Results

3.4.1 Effect of prey densities and photoperiods

There was a significant interaction between larval stage*light regime*prey density (ANOVA; df = 6, F = 7.77; $P < 0.001$). Feeding responses under either 0 h or 24 h of light differed according to prey density (Figure 1). Zoeae I average prey consumption was higher under the lowest and highest prey densities when exposed to 24 h of light (19.0 ± 0.7 and 112.0 ± 18.6 prey consumed, respectively) than in complete darkness (14.4 ± 0.3 and 96.0 ± 7.4 prey consumed, respectively). Under the three highest prey density, zoeae I prey consumption was highest under 12 h of light. Concerning zoeae II, average prey consumption under 24 h of light was the same as in complete darkness under all three lowest prey density but was lower (83.6 ± 8.6 and 120.8 ± 13.1) at the highest prey concentration. Under all tested feeding densities, prey consumption of larvae at the second zoeal stage was significantly higher under 12 h illumination (17.2 ± 2.7, 34.2 ± 2.8, 99.0 ± 4.3 and 142.8 ± 4.1 prey consumed for larvae fed at 0.5,

1, 3 and 5 prey ml^{-1}, respectively; Figure 1). Other general trends can also be observed, such as a significant overall increase of consumed prey with increasing food levels (from 15.6 ± 3.2 to 112.9 ± 21.7, df = 3, F = 1750.34; $P < 0.001$), for both larval stages and light regime experienced combined (Figure 1). Additionally, prey ingestion for both zoeal stages varied significantly with light conditions (df = 2, F = 35.38; $P < 0.001$), but was identical for both stages under the same light regime (df = 1, F = 2.92; $P = 0.91$).

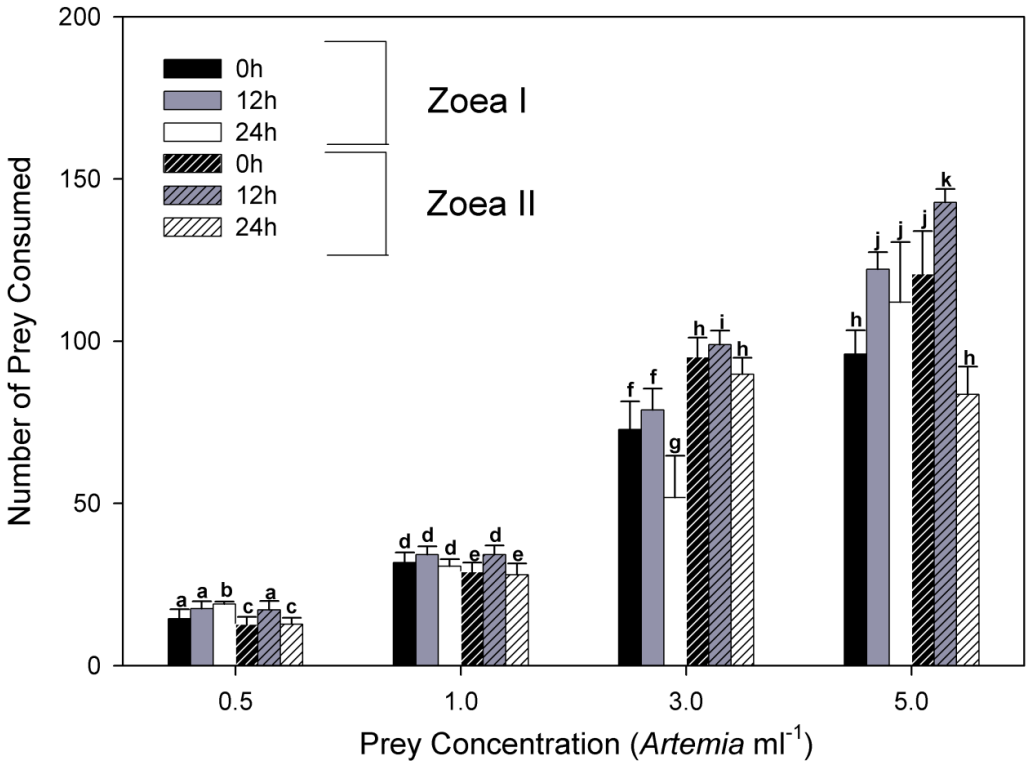

Figure 3.1: Prey consumption of *N. norvegicus* ZI and ZII under different prey densities and light conditions after a 24 h period. Prey concentration is expressed as the number of prey ml^{-1}. Light conditions are expressed as hour of illumination, i.e. constant darkness (0h), 12:12 light:dark photoperiod (12h) and constant illumination (24h). Error bars represent standard error. Different letters represent significantly different feeding rates.

3.4.2 Effect of prey types and prey densities

A significant interaction between larval stage*prey type*prey density was observed (ANOVA; df = 9, F = 22.2; $P < 0.001$). Regarding the first zoea, at low prey density, the number of prey ingested did not differ across prey types (17.6 ± 2.2, 15.0 ± 0.7, 13.4 ± 1.3, and 12.8 ± 0.8 for Naup, Meta, MetaAlga and MetaSpi, respectively; Figure 2). However, as prey density increased, prey consumption of metanauplii was reduced.

For a prey concentration of 1 prey ml^{-1}, consumption of Naup and Meta were identical but were higher than consumption of MetaAlga and MetaSpi. Finally, for the two highest prey densities, significantly more Naup were consumed than Meta which was, in turn, consumed in higher quantities than MetaAlga and MetaSpi ($P < 0.001$). There was no significant difference ($P > 0.972$) in the number of ingested enriched metanauplii at all tested prey densities (Figure 2). Concerning Zoea II, number of prey ingested did not also differ among prey types when these were provided at the lowest concentration (17.2 ± 2.7, 19.4 ± 0.9, 19.2 ± 0.8, and 19.2 ± 0.8 Naup, Meta, MetaAlga and MetaSpi, respectively; Figure 2). However, under the two highest prey densities consumption of Naup was always significantly lower ($P < 0.001$) than that of all metanauplii. Under all tested prey densities, consumption of Meta, MetaAlga and MetaSpi was always similar ($P > 0.999$) (Figure 2). Finally, larval feeding ability was significantly different between both zoeal stages, with first zoeae consuming less prey than second zoeae (stage; df = 1, F = 3802.3; $P < 0.001$).

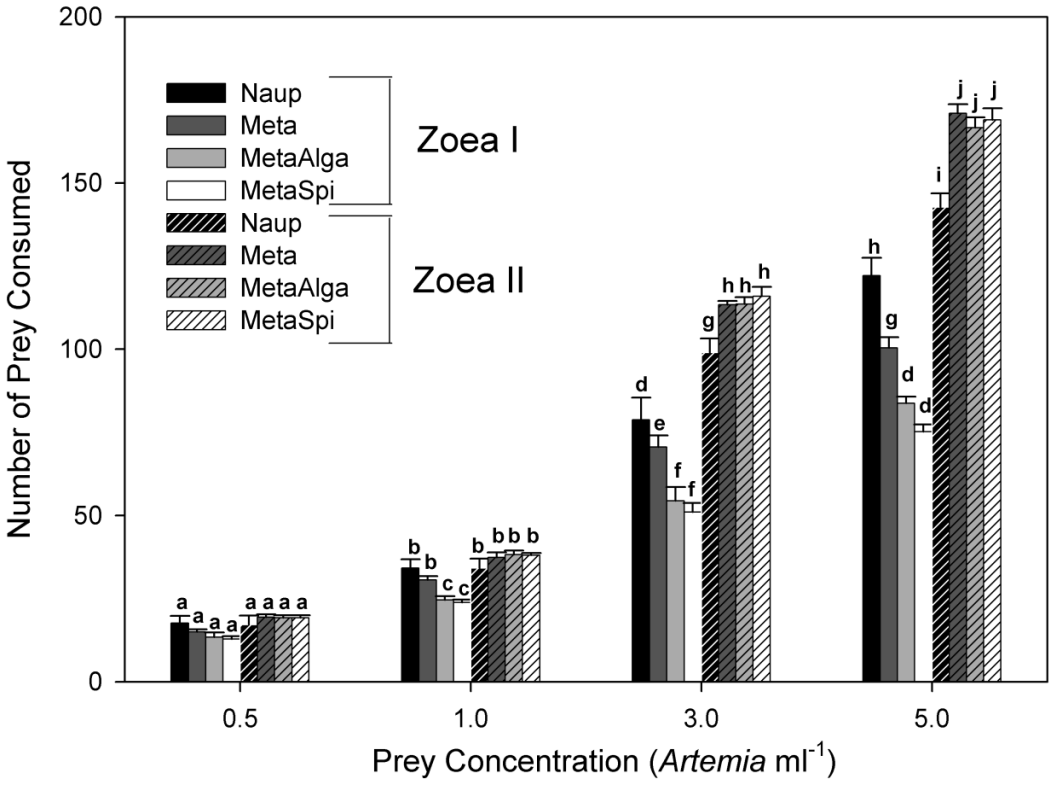

Figure 3.2: Prey consumption of *N. norvegicus* ZI and ZII under different prey densities and prey type after a 24 h period. Prey concentration is expressed as the number of prey ml^{-1}. Tested prey types were *Artemia* nauplii (Naup), metanauplii (Meta), metanauplii enriched with Algamac™ 2000 (MetaAlga) and Spirulina (MetaSpi). Error bars represent standard error. Different letters represent significantly different feeding rates

3.4.3 Effect of previous feeding histories

A significant effect of feeding history was recorded for both stages (ANOVA, Z1:df = 5, F = 1116.13; $P < 0.001$; Z2: df = 5, F = 1873.40; $P < 0.001$). Larvae of either stage previously starved for 12 h always consumed more prey than those allowed to feed under higher or lower prey densities during the acclimation period (Figure 3). However, exposing larvae to a higher (5 prey ml^{-1}) or lower (0.5 prey ml^{-1}) prey concentration during the acclimation period promoted different feeding responses by larvae subsequently placed under high or low food conditions (Figure 3). Zoeae acclimated to low and high food concentrations did not show differences in the number of prey consumed ($P > 0.999$) when subsequently placed under low food concentrations (8.8 ± 0.4, and 9.2 ± 0.8 Naup consumed by ZI and 8.8 ± 1.3 and 9.0 ± 1.0 MetaAlga consumed by ZII, respectively). In contrast, larvae acclimated to low food conditions consumed significantly more prey ($P < 0.001$) than those acclimated to high food densities, when subsequently placed in high food conditions (69.4 ± 2.4 and 56.6 ± 4.7 Naup consumed by ZI, and 97.2s ± 4.1 and 84.4 ± 3.8 MetaAlga consumed by ZII, respectively; Figure 3).

3.5 Discussion

In this study, larval prey consumption increased with prey density (from 0.5 to 5 prey ml^{-1}). The number of prey ingested did not reach a plateau suggesting that maximum prey consumption was not reached and would probably only be attained at higher prey densities. Successful culture of *N. norvegicus* larvae to the juvenile stage is possible using a diet of enriched *Artemia* sp. metanauplii, although survival to metamorphosis is always low (Anger and Puschel 1986; Dickey-Collas et al. 2000b; Figueiredo and Vilela 1972). In the present study it is possible that larval energetic requirements were not fulfilled. A lack of satiation is consistent with the observed linear increase in the number of ingested prey and supports the general belief that *Artemia* sp. is an inferior diet when compared to natural plankton (e.g. copepods), (Helland et al. 2003; Shields et al. 1999; Sorgeloos et al. 1998). As a result, energetic

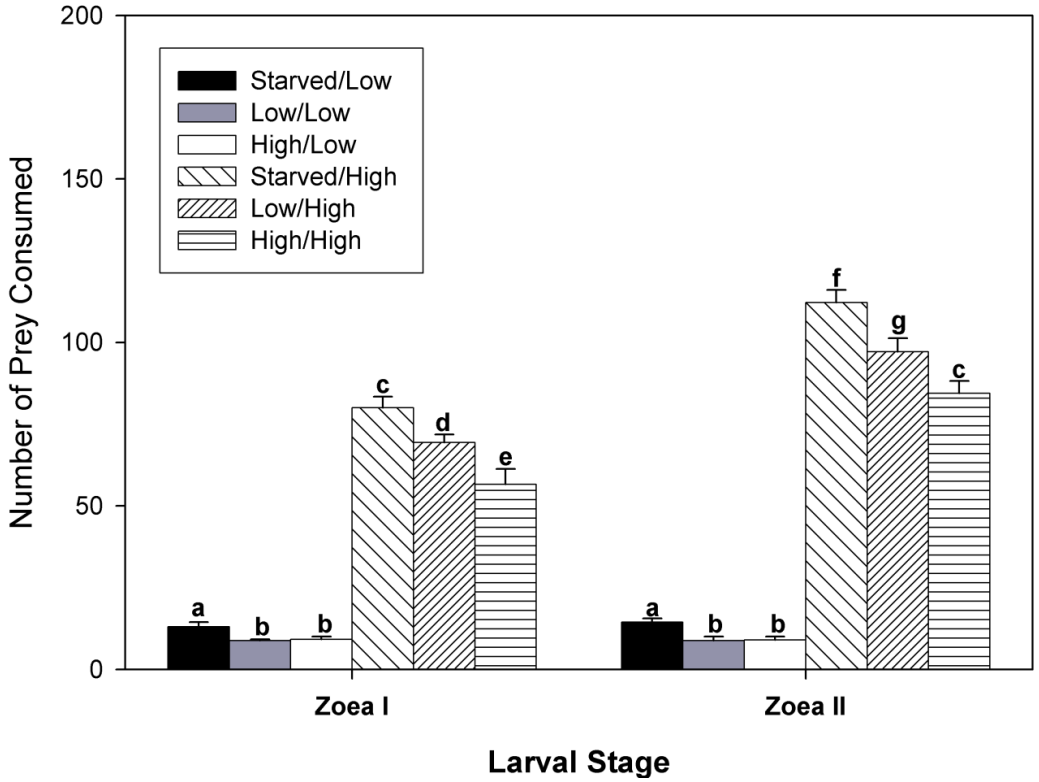

Figure 3.3: Prey consumption of *N. norvegicus* larvae under different prey densities previously acclimated to different prey densities after a 12 h period. The right (A) and left (B) figure depict the number of *Atremia* sp. consumed by Zoea 1 and Zoea 2, respectively. Low and high prey concentration represent densities of 0.5 and 5.0 *Artemia* sp. ml^{-1}, respectively. Zoea I and II were offered nauplii and metanauplii, respectively. Error bars represent standard error. For each zoeal stage separately, different letters represent significantly different feeding rates.

needs were not met. In this way, if the present study had been performed using natural food items the tested prey densities may have promoted different results and food intake at higher densities would have stabilize at a saturation prey density. However, further experiment is needed to support this hypothesis.

Although consumption under low prey density was not affected by prey type or larval stage, larval ingestion under higher prey densities was influenced by these two factors. Concerning ZI, the smaller size and inferior swimming speed of *Artemia* sp. nauplii can explain the higher consumption recorded for this type of prey, when compared to metanauplii. The lower consumption of enriched metanauplii, in comparison to unenriched ones, was probably due to their higher nutritional value, since unenriched and enriched metanauplii displayed similar sizes and swimming speeds. Feeding on enriched metanauplii may have caused ZI to reach satiation faster than when ingesting

a similar number of less nutritive unenriched metanauplii. Therefore, the present results suggest that the nutritional value of food items in a plankton patch will directly influence the number of prey ingested by ZI of *N. norvegicus*. The quality of a prey item is likely to vary with its nutritional state and this in turn affects larval feeding on that item (Dalsgaard et al. 2003). Therefore, larvae at the same development stage (thus with identical predatory ability) will capture and ingest different numbers of the same dietary item dwelling in a plankton patch, as a function of its nutritional value.

Regarding ZII, these larvae clearly displayed a preference for larger prey, eating significantly more metanauplii than nauplii. The small sized *Artemia* sp. nauplii did not trigger a proper predatory response and promoted lower prey consumption. In larval decapods it is not unusual to record a shift in larval prey's preference during development, with early stages preferring smaller food items, while later stages switch towards larger prey (McConaugha et al. 1991). Nonetheless, larger stages continue to ingest smaller prey if these are available (Harvey and Epifanio 1997), although larvae become increasingly selective at higher prey densities (Yúfera et al. 1984). In opposition to ZI, larvae in the second zoeal stage ingested similar numbers of enriched and unenriched *Artemia* sp. metanauplii. This similar feeding response to metanauplii may have been promoted by the fact that with neither prey types the larvae were able to reach satiation. Apparently, *N. norvegicus* ZII require larger and/or more nutritious dietary prey. In the wild this feeding response will result in a lower energetic intake if larvae dwell in a plankton patch dominated by prey with "low nutritional quality". It is possible that numerous dietary prey types can be available in a plankton patch but that the one available at the highest concentration is not nutritionally balanced to support optimal zooplankton growth. Ultimately, these prey may occur at high concentration because they are avoided by predating larvae (Mitra and Flynn 2006b). At least under culture conditions, the nutritional inadequacy of prey items can result in partial starvation and promote lower growth and survival (Harvey and Epifanio 1997). If developing *N. norvegicus* larvae are regularly exposed to suboptimal dietary prey, which they will ingest to avoid starvation, the resulting nutritional stress may still induce larval mortality. Therefore, in order to develop successfully, larvae must

regularly have at their disposal not only suitable numbers of dietary items, but also nutritionally balanced prey.

Zoeal stages of decapods are not capable of long periods of sustained horizontal swimming and may not be able to remain in food patches (Harden Jones 1980). This constraint will probably impair larvae to feed continuously throughout their ontogenic development (Pitchford et al. 2003). In the present research, early stage *N. norvegicus* larvae increased the number of prey consumed after previously experiencing food deprivation or low prey abundance. These feeding behaviors may allow *N. norvegicus* larvae to overcome intermittent periods of starvation, or suboptimal feeding levels, caused by the natural patchiness of plankton and abundance of suitable prey. A similar reaction would be expected in response to changes in food conditions due to variable prey composition caused by diel vertical migration of zooplankton (Forward 1988).

Although both zoeal stages displayed distinct prey preferences, larvae readily ingested all types of tested prey, even if these were "suboptimal" (e.g. the consumption of Artemia sp. nauplii by ZII). Adjacent plankton patches are not always similar in their species composition, and these may vary in terms of prey shape, size, quantity and quality (Petrone et al. 2005). The plasticity displayed by *N. norvegicus* larvae, in terms of prey types ingested, may therefore be an important adaptation to pelagic life. Even though N. norvegicus larvae yolk reserves may differ spatially and seasonally (Rotlland et al. 2004), they do not exhibit primary lecithotrophy, being unable to advance to Zoea II in the absence of food (personal observation). In that sense, and as suggested by modeling studies (Gentleman et al. 2003; Mitra and Flynn 2006a), the capability to maximize food intake when prey are available, regardless of their type or quality, can temporarily reduce the effects of starvation in oligotrophic winter conditions until suitable prey are available. In contrast, when the hatching of *N. norvegicus* occurs at the time of the spring bloom, and prey items are more abundant and varied (dos Santos et al. 2007), adequate prey are more likely to be available for newly hatched and developing larvae. In that sense, optimal foraging theory would predict that a predator adopts the feeding behavior that generates the highest intake rate of energy, with the consumption of suboptimal prey being highly reduced (Kiørboe et al. 1996). However, no study has ever addressed the existence of potential morphological and

behavioral constraints in *N. norvegicus* larvae that may mask and/or preclude optimal foraging. In this way, the present results and those from previous laboratorial studies addressing the feeding of Norway lobster larvae should be extrapolated with caution to a pelagic environment with complex plankton patches dynamics.

Larval feeding ability was affected by photoperiod duration, with the number of ingested prey being consistently superior when 12 h of light were employed, regardless of prey density or larval stage. However, responses to light may vary significantly during larval development of decapods (Bermudes and Ritar 2008). Several studies on crustacean larvae (Calado et al. 2008; Gardner and Maguire 1998; Starkweather 1976; Teschke et al. 2007), and especially spiny lobsters (Bermudes and Ritar 2008; Bermudes et al. 2008; Mikami and Greenwood 1997), have demonstrated that food intake, survival and molt stage duration are positively impacted by the presence of any kind of periodic light regime. Although decapod larvae are good swimmers, true hunting behaviors (e.g. prey chasing) are not displayed and these organisms solely rely on chance encounters with dietary prey (Epelbaum and Borisov 2006; Gonor and Gonor 1973). This feeding behavior has been termed by Berkes (1975) as “encounter feeding”. Since swimming activity of many decapods increases in the presence of light (Forward et al. 1984; Sulkin 1984), larvae will have increased chances of encountering dietary prey. However, constant illumination will increase energy expenditure and therefore reduce survivorship since larvae remain permanently active in the laboratory (Calado et al. 2008; Dawirs 1982). Although under constant darkness feeding remained possible, despite a lower consumption rate, 24 h of light did not promote higher prey ingestion than 12 h of light. These results seem to indicate that *N. norvegicus* larvae may also feed during the dark:light interface, as already suggested for spiny lobsters larvae (Bermudes and Ritar 2008). Apparently, the occurrence of a light:dark phase optimizes larval growth and feeding performances, regardless of the duration of the light phase, suggesting that the transition between day and night plays a greater role rather than the duration of the photoperiod itself (Bermudes and Ritar 2008; Bermudes et al. 2008; Starkweather 1976).

In conclusion, the results of the present study suggest that unsatiated *N. norvegicus* larvae are able to increase prey ingestion in the presence of plankton patches with

higher food abundance. The feeding plasticity indicates that early stage larvae may be able to withstand and recover from periods of suboptimal food conditions by switching prey preference and increasing consumption once optimal foraging conditions are encountered. Both prey availability and prey size play a crucial role in the trophodynamics of developing *N. norvegicus* larvae. Extreme photoperiods resulted in lower prey consumption for both larval stages and their use in *N. norvegicus* larviculture is not recommended.

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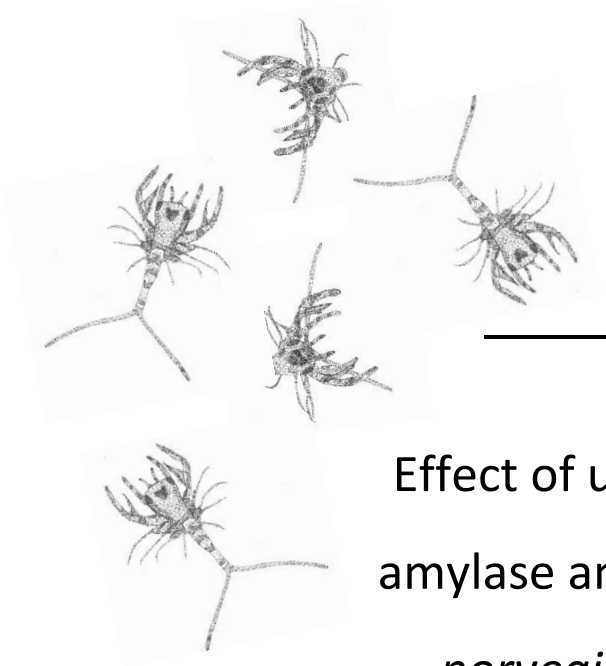

Chapter 4

Effect of unfavorable trophic scenarios on amylase and protease activity of *Nephrops norvegicus* (L.) larvae during their first vertical migration: a laboratory approach

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Chapter 4

Effect of unfavorable trophic scenarios on amylase and protease activity of *Nephrops norvegicus* (L.) larvae during their first vertical migration: a laboratory approach

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Key words - *Nephrops norvegicus*, Decapod crustacean larvae, Digestive enzymes, Fluorometry, Starvation

4.1 Abstract

In Portuguese waters, *Nephrops norvegicus* larvae hatch at 400-800 m depth and need to perform a vertical migration to food-rich shallower waters to find suitable prey. The effect of suboptimal feeding on digestive enzymes activity of *N. norvegicus* larvae during this early period of their larval life remains unknown. Protease and amylase activity was investigated ex situ using flurometry in laboratory hatched larvae exposed to different feeding and/or starving scenarios in the 24 h following hatching, the period during which they typically accomplish their upward vertical migration. Amylase activity was very low in comparison to protease activity, indicating that carbohydrates are not a primary energy reserve. Larvae starved for 12h and subsequently fed displayed no increase in amylase activity, which suggests that feeding may be required before 12h post-hatch to trigger amylase activity. Protease activity was high under all feeding conditions and the increase of protease activity under sustained starvation indicated the catabolism of protein reserves. The ability of first stage *N. norvegicus* larvae to metabolize protein reserves may play a decisive role for their survival during their first vertical migration, as it enables them to overcome the deleterious effects of short-term starvation and/or suboptimal feeding.

4.2 Introduction

The Norway lobster, *Nephrops norvegicus* (Linnaeus, 1758), is a commercially important benthic decapod crustacean commonly found in Northeastern Atlantic waters, from the Coast of Iceland to Morocco, and in the Mediterranean Sea (d'Udekem d'Acoz 1999). After embryonic incubation on the female abdomen, larvae are released from the eggs, which then develop through three zoeal planktonic stages, each intermolt period lasting about 10 days at 10ºC (Figueiredo and Vilela 1972), and then migrate back to the bottom as a post-larva before settling on the benthos as a juvenile. The adult depth range varies from 15 to 800 m, although they are typically found on the NE Atlantic coast between 300 and 600 m depth (Tuck et al. 1997a) and

200 and 800 m in the Mediterranean (Maynou and Sardà 1997). In addition to differences in the depth range, the reproduction period of this species also varies with latitude, as average embryo incubation period lasts up to 10 months in the Northeastern Atlantic and only 6 months in the Mediterranean Sea (Sardà 1995). These latitudinal differences affect the timing of larval hatching in *N. norvegicus* , with larval release occurring by early spring in the Northeastern Atlantic and at the end of winter in the Mediterranean (Rotllant et al. 2004). Off the Coast of Portugal, adult Norway lobsters occur at depths ranging from 400 to 800 m and larval hatching period is known to take place from December to April (dos Santos and Peliz 2005). In this way, while larvae hatching at the end of winter will face a nutritionally poor environment, larvae hatching a few months later will overlap with the beginning of thermal stratification and the spring bloom [chlorophyll *a* peaks, and therefore zooplankton blooms (dos Santos et al. 2007; Moita 2001)].

Apart from seasonal variations, the depth where larval hatching takes place may also play a role in food availability. *N. norvegicus* larvae hatch below 300 m depth off the Portuguese coast (dos Santos and Peliz 2005) and at these depths larval prey may not be available to fulfill the nutritional needs of first stage *N. norvegicus* larvae. In fact, it is possible that suitable prey may only be available once these larvae reach the upper regions of the water column (Labropoulou and Kostikas 1999). After hatching, *N. norvegicus* larvae are known to migrate vertically to the photic zone, as stage I larvae have already been recorded in Portuguese waters at depths as low as 60-70 m (dos Santos and Peliz 2005). This aspect of *N. norvegicus* early life cycle may translate into a potential period of suboptimal feeding, or even post-hatching starvation.

Larvae of *N. norvegicus* are capable of maximizing food intake when prey are available, regardless of their type or quality, and therefore can temporarily attenuate the deleterious effects of starvation (Pochelon et al. 2009a). Although the analysis of late stage embryos of homarid lobsters has revealed that newly hatched larvae still contain considerable energetic reserves (Pandian 1970a, b; Rosa et al. 2005; Rosa et

al. 2003), these organisms may not recover from nutritional stress if suitable prey are not available soon after hatching (Anger et al. 1985; Rotllant et al. 2001).

In this way, the study of digestive enzymes can help clarify how first stage larvae of *N. norvegicus* cope with the potential absence of suitable prey at great depths, as well as during their ontogenetic migration towards food-richer environments in ocean upper layers. Digestive enzymes dynamics during periods of starvation (or suboptimal feeding) indicate which nutrients act as energy reserves and which are preferentially catabolized (Johnston 2003; Johnston et al. 2004; Jones et al. 1997; Kamarudin et al. 1994; Lovett and Felder 1990b; Rotllant et al. 2010). For instance, high protease activity is indicative of protein catabolism, while amylase activity indicates the consumption of carbohydrates (Johnston 2003; Kamarudin et al. 1994). It is not consensual whether proteins play the major role as energy sources to fuel decapod larvae during starvation (Anger 2001; Johnston et al. 2004), since lipids also appear to be catabolized when larvae are deprived of food (Ritar et al. 2003a). However, lipase activity has been shown not to be significantly affected during either feeding or starvation of the first larval stage of nephropid lobsters (Biesiot and Capuzzo 1990a, b). The nutritional relevance of carbohydrates for decapods crustaceans is still not fully understood, as marine decapods are not able to efficiently store carbohydrates reserves (Sánchez-Paz et al. 2006). Nonetheless, it has been suggested that amylase may play other metabolic roles, which still need further investigation, than simply catabolizing carbohydrates (Jones et al. 1997). By studying amylase activity in carnivorous larvae, it may be possible to understand why this enzyme is commonly active in these organisms in spite of the scarcity of its substrate.

So far, no study has ever investigated the shifts in digestive enzyme activity of *N. norvegicus* larvae exposed to various periods of starvation and/or feeding during their first hours of life, during which they perform their first vertical migration. In this way, the objective of the present research was to determine through laboratory experiment the protease and amylase activity in first stage *N. norvegicus* in response to extended and intermittent periods of starvation and feeding (lipase activity was not

considered in the present study, as it is known not to be significantly affected by food intake in the first larval stage of other nephropid lobsters; Biesiot and Capuzzo 1990a; Biesiot and Capuzzo 1990b). The experimental feeding regimes selected in the present work aim to simulate different feeding scenarios that *N. norvegicus* larvae may experience during their vertical migration from deeper to shallower waters.

4.3 Materials and Methods

4.3.1 Larval production

Ovigerous *N. norvegicus* were collected between February and April 2008 using commercial baited traps off Peniche (West Coast of Portugal), at an approximate depth of 400 m, and stocked in the laboratory. Ovigerous females were held in individual aquaria (0.4 m x 0.2 m x 0.3 m ≈ 24 L) placed in a temperature controlled room (15±1ºC), until their larvae hatched. Larval release always occurred within a week from capture and females were therefore not fed prior to hatching. Natural seawater was used, at a salinity of 35. Seawater was 1 µm filtered, passed through activated carbon and UV irradiated before being used. About one third of the volume of each broodstock aquarium was replaced by new filtered seawater every other day.

4.3.2 Larval feeding trials

Within one hour of hatching, *N. norvegicus* larvae were removed from the aquaria using a plastic cup and randomly distributed across the eight experimental trials and a control. Only those specimens displaying strong phototactic responses were selected for the experimental trials, since this behavior is commonly employed in crustacean aquaculture to evaluate larval quality (see Treece and Fox 1993). Larvae were sampled for later enzyme analysis at the end of a set period under predefined conditions. Those included, a control group of newly hatched (NH) larvae sampled immediately after hatching, larvae collected 6, 12 and 24 h after hatching in either continuous starvation or feeding (S6, S12, S24, F6, F12 and F24, respectively), and two “mixed” treatments where larvae were sampled 24 h after being initially starved for 12 h and

fed on the following 12 h; or being initially fed for the first 12 h and subsequently starved in the next 12 h (S12F12 and F12S12, respectively). For each of the nine groups, 15 larvae were placed individually in 40 mL plastic beakers with filtered seawater at a salinity of 35 in a climatized chamber at 15±1ºC under constant darkness (to prevent larval prey from grouping at the surface of the beaker due to their positive phototaxis). Larvae in the feeding treatments were provided with newly hatched Artemia sp. nauplii (San Francisco Bay Brand Inc., Newark, CA, USA) at a density of 5 nauplii mL-1, with Artemia sp. cysts being incubated according to the procedures described by Sorgeloos et al. (1998). To ensure a homogenized distribution of prey in the water column during the experimental trials, each plastic beaker was gently aerated at a rate of ca. one air bubble per second.

At the end of each experimental trial, larvae were stored for later enzyme analyses, as described by Rotllant et al. (2008). In brief, larvae were gently dried on filter paper and individually placed in a 1.5 mL Eppendorf before being freeze-dried and stored at -32ºC for later analysis. Prior to enzymes analysis, each larva was homogenized in 100 µL of distilled water and sonicated in an ice bath with three short pulses of 2 s (Vibra-cell., Sonics, USA). The homogenate was centrifuged for 5 min at 12,000 rpm at 4ºC. The supernatant was kept on ice and used for the analysis of amylase and protease activity, as well as larval protein content.

4.3.3 Amylase activity

The homogenate to quantify amylase activity was diluted to 1:50 in the case of larvae fed for 6, 12 and 24 h, and 1:10 for all other treatments. From this diluted sample 50 µl were added to a microplate along with in 50 µl of a working substrate (prepared following the manufacturer's protocol "EnzCheck® Ultra Amylase Assey Kit" [E33651; Molecular Probes®]) was used for the analysis. Fluorescence was measured at 485 nm (excitation) and 535 nm (emission) for 10 min at 30ºC. Samples were assayed in duplicates.

4.3.4 Protease analysis

To quantify protease activity 100 µL of Tris-HCl 0.1 M pH 8.0 and 10 µL of fluorescent casein (C-2990_25mg; Molecular Probes®) were added to 10 µL of the homogenate and incubated for 1 h at 37ºC. The reaction was then stopped by adding 30 µL of TCA 20%. The reaction was centrifuged for 5 min at 12500 rpm at 4ºC and 50µL of the supernatant was added to a microplate along with 200 µL of Tris-HCl 0.5M pH 8.5. Fluorescence was measured at 485 nm (excitation) and 538 nm (emission) for 20 ms at 30ºC. Samples were assayed in duplicates. Additionally, a blank was conducted for each sample where the homogenate was added to the substrate and buffer after the reaction has been stopped. The measured value of the blank was then subtracted to correct the value of the samples (Rotllant et al. 2010).

4.3.5 Protein analysis

In order to standardize enzymatic activity data, the latter was expressed as specific activity (RFU/min/µg prt). Protein content was measured in the homogenates as described by Bradford (1976) using Bio-Rad Protein Assay dye reagent (BioRad 500-0205) and bovine serum albumin (BSA, Sigma A7906) as the standard. Samples were assayed placing 15 µL of the homogenate in a 48 flat bottom transparent microplates with 985 µL Bio-Rad Protein Assay dye reagent (BioRad 500- 0205) and read at 595 nm

4.3.6 Statistical analysis

Differences between each treatment in both per individual and specific enzymes activity were compared. Given that all larvae survived through the experimental period, the number of larvae employed on enzymatic analysis was identical to the ones used on each feeding trial (n=15). Due to the non-normality of the data a Kruskal-Wallis test was used (Zar 1999) followed by a non-parametric post-hoc tests comparing mean rank of all pairs of groups (Siegel and Castellan 1988). All tests were performed using STATISTICA 8.0 (manufactured by StatSoft Inc., USA).

4.4 Results

Total amylase activity of first stage *N. norvegicus* larvae varied significantly with the feeding regime (Kruskal-Wallis test, H_{138} =85.1, P<0.001). Total amylase activity was not significantly different (post-hoc tests, P>0.24) between newly hatched (NH) and permanently starved larvae (S6, S12 and S24), nor from that displayed by larvae under fed/starved treatments (S12F12 and F12S12) (Figure 1a). A significant increase in total amylase activity was however observed in constantly fed larvae (post-hoc tests, P<0.009). After 6 h of feeding, amylase total activity was similar to that recorded for larvae in the F12S12 treatment (post-hoc tests, P>0.9). Highest total amylase activity was found after 12 h or more of continuous feeding, with both F12 and F24 larvae displaying significantly higher activity than all other groups (post-hoc tests, P<0.03), with the exception of F6 larvae (post-hoc test, P>0.9). Finally, larvae in the S12F12 treatment displayed levels of total amylase activity similar to those of starved larvae (post-hoc tests, P<0.48) and significantly lower than that of F12 larvae (post-hoc test, P>0.03).

Total protease activity displayed by larvae exposed to different feeding regimes was more variable (Figure 2a), although significant differences were still recorded (Kruskal-Wallis test, H_{143}=39.37, P<0.001). There was no significant difference between NH, starved larvae (S6, S12 and S24), F6 and both fed/starved treatments (S12F12 and F12S12; post-hoc tests, P>0.39). Additionally, total protease activity was similar for larvae in the S24, F6, F12, F24, S12F12 and F12S12 feeding treatments (post-hoc tests, P >0.9). With time, a slight, however not statistically significant increase in total protease activity was observed either in constantly fed (F6, F12 and F24) or constantly starved (S6, S12 and S24) larvae.

Results of statistical analysis of the specific protease and amylase activity was almost identical to per individual activity, indicating that soluble protein content was constant among most experimental treatments (Figure 1b and 2b).

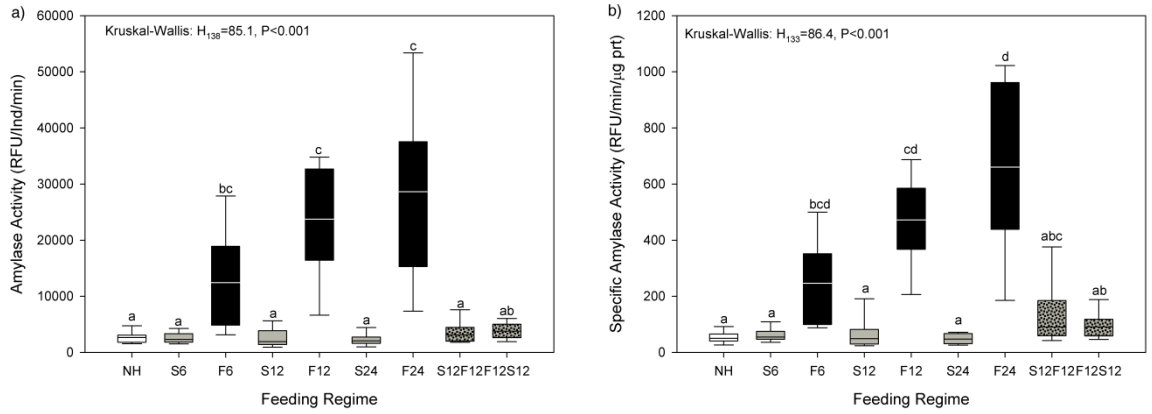

Figure 4.1: *Nephrops norvegicus*: Boxplot depicting a) total amylase activity (RFU/Ind/min) and b) specific amylase activity (RFU/min/μg prt) for first stage larvae under different feeding regimes (n=15). The white, grey, black and checked square boxes represent newly hatched, starved, fed and mixed treatments, respectively. The line in each box indicates the median and 50% of the ratings have values within the box. Error bars represent range. Different letters represent significantly different amylase activity.

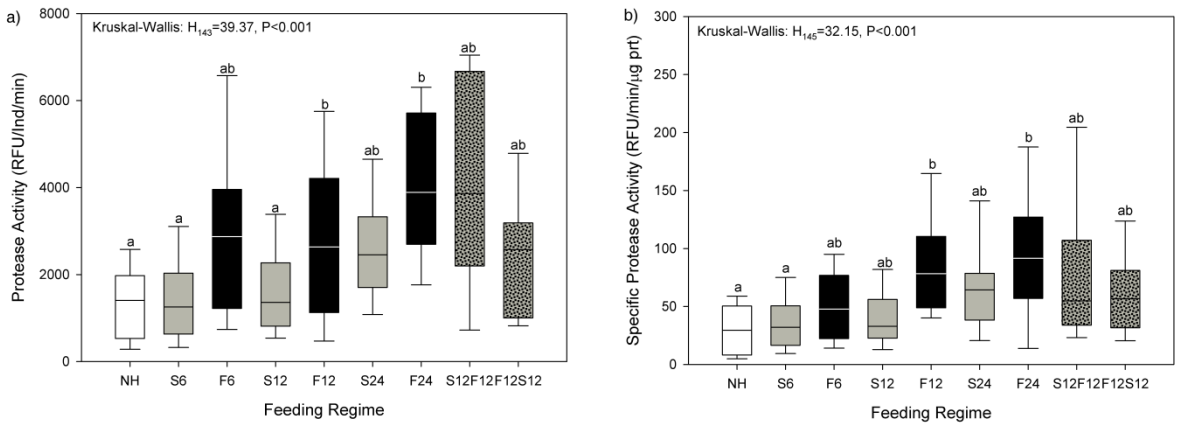

Figure 4.2: *Nephrops norvegicus*: Boxplot depicting a) total protease activity (RFU/Ind/min) and b) specific protease activity (RFU/min/μg prt) for first stage larvae under different feeding regimes (n=15). The white, grey, black and checked square boxes represent newly hatched, starved, fed and mixed treatments, respectively. The line in each box indicates the median and 50% of the ratings have values within the box. Error bars represent range. Different letters represent significantly different protease activity.

4.5 Discussion

In the present study, digestive enzyme activity was significantly affected by the feeding regime experienced by first stage *N. norvegicus* larvae. Amylase activity was significantly lower in first stage larvae experiencing food deprivation, either

continuously or semi-continuously ("mixed" treatments S12F12 and F12S12) than in the continuously fed treatments. As expected, these results confirm that carbohydrates are not the main energetic reserve fueling larval metabolism during periods of starvation. Additionally, the results of the S12F12 treatment indicated that larvae initially exposed to starvation do not recover amylase activity even after feeding, as those larvae exhibited significantly lower amylase activity than conspecifics continuously fed for 12 h. In this way, it is possible that the ingestion of carbohydrates by *N. norvegicus* immediately after hatching may be required to trigger "normal" amylase activity. If first stage larvae are exposed to a period of food deprivation, and consequently fail to trigger amylase activity, it is possible that those specimens will no longer be able to digest carbohydrates efficiently, at least on a short term basis. Since the present study only focused the first 24 h post-hatching, during which the first vertical larval migration is performed, further studies are needed to assess the long term effects of reduced amylase activity in developing larvae. Two outcomes are possible: 1) amylase activity will recover with long term sustained feeding or; 2) larvae will not be able to overcome the deleterious effects of early starvation and their survival will be negatively affected [as it is speculated that amylase may play other metabolic roles than simply catabolizing carbohydrates (Jones et al. 1997)]. The assumption of deleterious effects resulting from early larval starvation is supported by several works addressing other decapod species, on which first stage larvae are unable to successfully molt to the next larval stage if optimal feeding does not occur during the first 24-48 h (Calado et al. 2007a; Giménez 2002; Rotllant et al. 2010). Additionally, free carbohydrates, in the form of glucose, are known to play a critical role in energy metabolism, and carbohydrates are needed for the formation of the larval exoskeleton (Anger 2001). When larvae were continuously fed, there was a gradual increase over time in amylase activity. Results of the F12S12 treatment suggest that the carbohydrates are rapidly digested, as already recorded for the early larval stages of the spiny lobster *Jasus edwardsii* (Ritar et al. 2003a). Indeed, at the end of that treatment, amylase activity dropped back to levels similar to those of starved larvae.

All fed larvae, including the ones in the mixed treatments (S12F12 and F12S12), had higher levels of protease activity than NH, S6 and S12. However, larvae starved for 24 h exhibited levels of protease activity comparable to those observed for fed larvae. As larvae hatching at great depths are unlikely that to be able to feed abundantly before reaching the upper part of the water column (Yamaguchi et al. 2005), first stage *N. norvegicus* probably display some type of mechanisms that able them to cope with post-hatching short-term food deprivation. The present work revealed that protease is active immediately after hatching and that starved larvae display an increasing protease activity over time. High protease activity may enable *N. norvegicus* larvae to rapidly extract protein from food once it becomes available. This strategy is commonly exhibited by decapod larvae, as it allows them to maximize the energetic intake under scenarios of intermittent starvation caused by prey patchiness (Le Vay et al. 2001). In an oligotrophic environment, such as the deep sea, the ability of first stage decapods to rapidly and efficiently digest any available prey prior to their arrival to shallower waters would certainly be an advantageous trait.

Specific enzyme activity recorded for larvae exposed to different feeding regimes was similar to total enzyme activity. In the S24 treatment, specific protease activity was lower than in the S12 treatment which might indicate that protein reserves start being used to endure food deprivation. Nevertheless, the lack of difference between specific and absolute activity results, indicate that protein reserves are not significantly depleted after 24 h of starvation and suggest that *N. norvegicus* larvae can survive over 24 h without any exogenous protein supply. In a previous study, the soluble protein content recorded for first stage *N. norvegicus* larvae from Mediterranean and Irish Sea populations (Rotllant et al. 2004) were between 2 to 3 times higher than that measured in the present study for Portuguese larvae. This is not surprising, as regional differences have already been observed in embryonic lipid and protein content in various decapods (Bas et al. 2007; Rosa et al. 2007a; Silva et al. 2009). Additionally, brood variability in yolk reserves may also play a decisive role in the ability of first stage larvae to endure starvation. Brood variability has already been documented for other nephropid lobsters, such as *H. americanus* (Anger et al. 1985; Pandian 1970b)

and *H. gammarus* (Moland et al. 2010; Wickins et al. 1995). A preliminary study addressing *N. norvegicus* also revealed significant within brood variability on the fatty acid profiles of developing embryos (Pochelon et al. 2009b). This finding, coupled with laboratorial observations of larvae from a single brood hatching over several consecutive days [as already reported for *Homarus* spp. (Pandian 1970b; Wickins et al. 1995)], indicate that *N. norvegicus* larvae will probably also display variable energetic reserves at hatching. This aspect has not been investigated so far in *N. norvegicus* larvae and may be of paramount importance for the survival of first stage larvae during their first vertical migration from deep to shallower waters.

In summary, first stage *N. norvegicus* larvae off the Coast of Portugal, hatching between 400 and 800 m deep, need to perform a vertical migration through the water column towards food rich upper layers. The urgency of this behavioral response to avoid the negative effects of starvation is emphasized by the relatively lower protein reserves present in studied larvae, when compared to Mediterranean and Irish Sea conspecifics. The present results indicated that, even though there seems to be a deleterious effect of starvation on amylase activity, at least on a short timescale, post-hatching starvation does not seem to affect the onset of protease activity. If suitable preys are not ingested in 12-24 h post-hatching, larval protein reserves will start to be catabolized to fulfill energetic demands. Although lipase activity does not seem to be affected by post-hatching starvation in *H. americanus* (a shallow water nephropid lobster related to *N. norvegicus*) (Biesiot and Capuzzo 1990b), it has already been shown that several decapod larvae catabolize lipids during periods of food shortage [e.g.: the lobster *Jasus edwardsii* (Johnston et al. 2004; Ritar et al. 2003a), the anomuran crabs *Lithodes santolla* and *Paralomis granulosa* (Kattner et al. 2003) and penaeid shrimp (D'Abramo 1989)]. In this way, future studies should investigate if lipase activity during post hatching starvation in *N. norvegicus* follows the pattern identified for shallow water nephropid lobsters (no significant changes in lipase activity) or the one displayed by other decapod crustaceans (significant changes in lipase activity).

The long term consequences of suboptimal feeding immediately after hatching were not tested in the present study. Therefore, additional work is required to clarify if metamorphosis and post-settlement performance of *N. norvegicus* can be negatively affected by suboptimal feeding during the first vertical migration of larvae.

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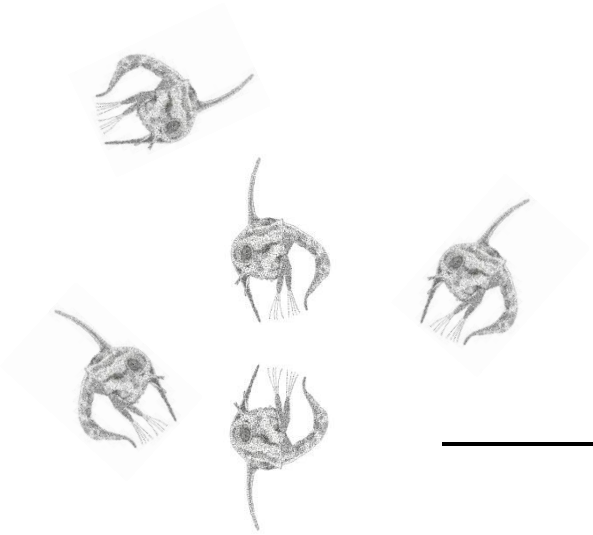

Chapter 5

Vertical larval distribution of an offshore brachyuran crab, *Monodaeus couchi*, off the South Coast of Portugal

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Vertical larval distribution of an offshore brachyuran crab, *Monodaeus couchi*, off the South Coast of Portugal

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Keyword: Crustacean, Invertebrate Larva, Offshore, Diel Vertical Migration, Vertical Distribution, *Monodaeus couchi*, Iberian Peninsula, South coast of Portugal.

5.1 Abstract

Knowledge of larval distribution and abundance is of major value in predicting the location and size of a breeding population. In this study, vertical distribution of the larvae of a brachyuran crab, *Monodaeus couchi*, was assessed during two week-long campaigns conducted at the end of January 2006 and 2007 off the South Coast of Portugal. Larvae were collected by oblique plankton hauls with a Longhurst-Hardy Plankton Recorder (LHPR) from the surface to 300 m. Abundance and distribution of zoea I and II were correlated during both years. For all stages, abundance decreased with depth during the day while it increased with depth at night; the larvae thus displayed reverse diel vertical migration. Abundance of zoea I and II was correlated with chlorophyll *a* levels, whereas those of later stages where correlated with neither physical parameters (chlorophyll *a*, temperature or salinity), nor with each other. An ontogenic shift in vertical distribution explained the results; earlier zoeal stages remain in the food-rich upper water column while later stages migrate to the bottom for settlement.

5.2 Introduction

Describing the distribution, abundance and dispersal of larvae of deep-sea animals is a daunting task. The small sizes of the larvae and the very large volumes of ocean into which they may potentially disperse present difficult logistical and technical constraints (Tyler and Young 1999). In one of the most successful field studies of deep-sea larval distribution, Mullineaux et al. (1995)used a MOCNESS system to sample a neutrally buoyant plume over the Juan de Fuca Ridge Larvae of vent gastropods and bivalves attenuated very rapidly outside the plume, illustrating the difficulty of sampling highly dilute larvae in the deep sea.

During two oceanographic cruises conducted off the south coast of Portugal in the winters of 2006 and 2007, we collected numerous larvae of the crab *Monodaeus couchi.* This brachyuran crab inhabits muddy substrata (Ingle 1983b) from England to

Angola and in the Mediterranean, over a bathymetric range extending from 60 to 1300 m (Gonzalez-Gurriarán and Méndez G. 1985). It is commonly associated with mud volcanoes, carbonate chimneys and cold seeps in the Gulf of Cadiz region (Cunha, M. R., University of Aveiro, unpublished observations). In slope habitats of the southern Iberian Peninsula and of the Mediterranean Sea, the species co-occurs with the commercially important Norway lobster *Nephrops norvegicus* (Gonzalez-Gurriarán and Méndez G. 1985; Maynou and Sardà 1997). Although not strictly a deep-sea species, it may provide a good model for the understanding some aspects of the larval ecology of deep-sea decapod crustaceans.

So far, field evidence on the factors that regulate dispersal of decapod larvae have been mostly based on studies of estuarine and coastal species (DeVries et al. 1994; DiBacco et al. 2001; Epifanio and Garvine 2001; Rothlisberg et al. 1995) and much less on offshore sampling programs (e.g. dos Santos and Peliz 2005; Lindley 1986). These studies, which include laboratory studies, demonstrate a commonality of behaviors with respect to feeding, predator avoidance, swimming and vertical migration (reviewed by Queiroga and Blanton 2005). Many life-cycle traits are phylogenetically conserved in deep-sea species (Eckelbarger and Watling 1995; Tyler and Young 1999; Tyler and Young 2003; Young 2003). Therefore, we would expect that at least some of the selective pressures and adaptations exhibited by larvae of shallow water decapods will also apply in general to deep-water species. Most decapod larvae are herbivores and/or carnivores (Anger 2001) and, therefore, must feed in surface layers. For deep-sea crab larvae a migration towards the surface would necessitate considerable energetic investment because crab larvae are negatively buoyant (Queiroga and Blanton 2005). However, crab larvae are relatively strong swimmers capable of sustaining velocities on the order of 0.5 to 2.0 cm s^{-1} ((Chia et al. 1984; Mileikovsky 1973), and could move over a vertical distance of 1000 m in a relatively short time. However, feeding in surface waters would expose them to a higher predation pressure than in the relatively protected environment of the deep-sea, and would require a return migration to deep waters at the end of planktonic life. Moreover, a distribution

in surface waters may also have considerable consequences for horizontal dispersal and for recruitment into appropriate habitats.

Information on larval distributions is rare for deep-sea crabs. In this short note we describe the distribution, abundance and vertical migration of *Monodaeus couchi* larvae in the Gulf of Cadiz. We show that the whole larval series is found in surface waters around the high chlorophyll *a* layer, which is a level commonly associated with high food availability (Verity et al. 2002) and that later stages are found at increasingly greater depths. Finally, we describe evidence for a reverse diel migration that may be related to predator avoidance (Han and Straskraba 2001).

5.3 Methods

5.3.1 Field collection

Plankton samples were collected during two oceanographic cruises conducted off the south coast of Portugal (Fig. 1a), one from January 29th to February 5th of 2006 (17 locations; day: n=7, night: n=10; Fig. 1b) and the other from January 24th to 31st of 2007 (9 locations; day: n=4, night: n=5; Fig. 1c). The sampling areas were located over the slope off the coast of Algarve, between Cape St. Maria and the Spanish border, over depths of 100 to 800 m (Fig. 1b, c). Samples were collected in oblique hauls with a Longhurst-Hardy Plankton Recorder (LHPR) equipped with a 42 cm cone, 280 μm mesh net and a flow meter. The water column was resolved into approximately 25 m depth strata, from the surface to 10 m above the bottom, or to a maximum depth of 300 m and 200 m in 2006 and 2007, respectively. In 2007, vertical profiles of temperature, salinity, chlorophyll *a* concentration and light intensity were measured with a Seabird SBE 9 plus CTD fitted with a Seapoint fluorometer and a Chelsea Instruments PAR sensor, which was deployed at each location immediately after collection of the plankton samples. Technical problems prevented the measurement of hydrological parameters in 2006. During both oceanographic cruises, the organisms sampled were fixed immediately in 4% buffered formaldehyde and brought to the

laboratory for taxonomic identification (Ingle 1983a). The larval series of *M. couchi* comprises 4 zoeae and 1 megalopae (Ingle 1983a). Since the number of megalopae was very low (only 1 larva caught in both years combined) this larval stage was not considered in the analysis. Abundances are reported as number of larvae per 100 m^{-3}.

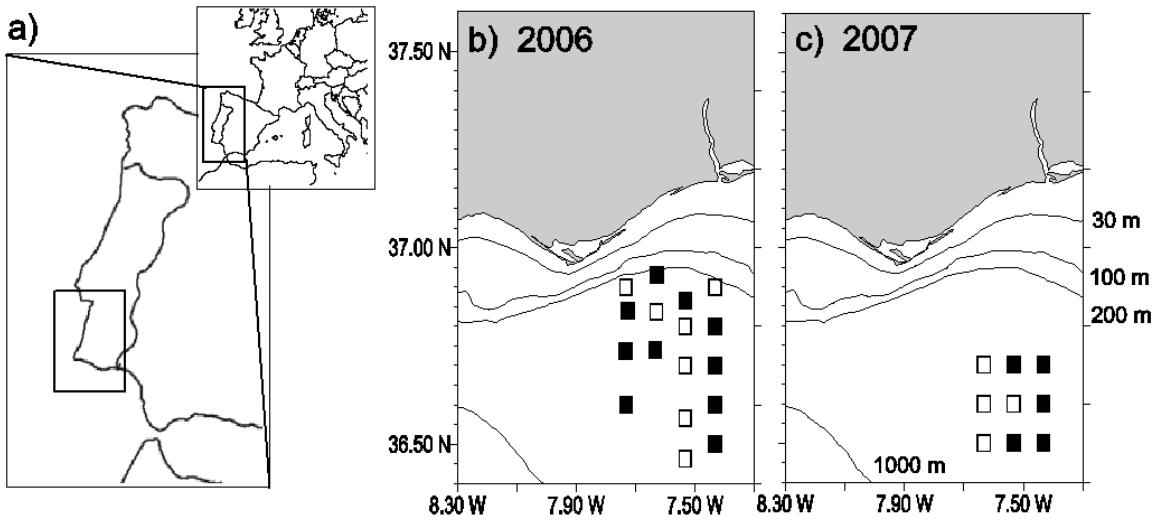

Figure 5.1: Map showing Europe and Portugal (a) and the locations of sampling sites in 2006 (b) and 2007 (c). b) Sampling started on January 29th, 2006 at 12:00 and ended February 1st, 2006 at 16:30. c) Sampling started at station 1 on January 24th, 2007 at 15:00 and ended January 31st, 2007 at 11:15. For both years, squares and circles represent sampling with LHPR and Bongo, respectively. Black and white indicate night and day sampling, respectively, while half colored represent two independent sampling taken during the day and at night.

5.3.2 Vertical distribution

In order to examine diel variation in catch of *M. couchi* abundance were pooled into 50 m intervals from the surface to 300 m or 200 m for 2006 and 2007, respectively. Due to the very low abundances of larvae caught in 2007 when compared to 2006, statistical tests were performed for each year separately. Zoea IV abundances were excluded from the analysis in 2007 because only one larva was caught that year. Abundances were classified according to time of the day (2 levels, day and night), larval stage (4 and 3 levels for 2006 and 2007, respectively) and depth (6 and 4 levels for 2006 and 2007, respectively) and differences were assessed using a 3-way

orthogonal PERMANOVA. All statistical tests were performed with Primer 6.1 with PERMANOVA add-on (Primer-E Ltd, Plymouth, UK). All PERMANOVAs used a type III sum of squares, 9999 permutations and permutation of residuals under a reduced model. Data were transformed [log(a+0.5)] before computation of the resemblance matrix using Euclidian distances. Whenever results were significant (P<0.05) a post-hoc comparison test was used. In order to further describe differences in depth distribution with respect to ontogenetic development, the mean depth of each stage was computed for each year using a weighted average accounting for differential larval abundances at each depth.

5.3.3 Correlation between larval stage and environmental parameters

To assess if there was a relationship among the distribution and abundance of larval stages in each year, we performed correlation analyses among abundances of the different stages. For these analyses, sampling sites with neither larval stage (double zeros) were excluded. Because multiple comparisons were performed, the significance levels were adjusted using a sequential Bonferroni correction. Additionally, the correlation between larval abundance and environmental parameters (temperature, salinity and chlorophyll *a* concentration) was assessed for 2007. Due to the non-normality of the data, a non-parametric Spearman correlation test was performed in all cases.

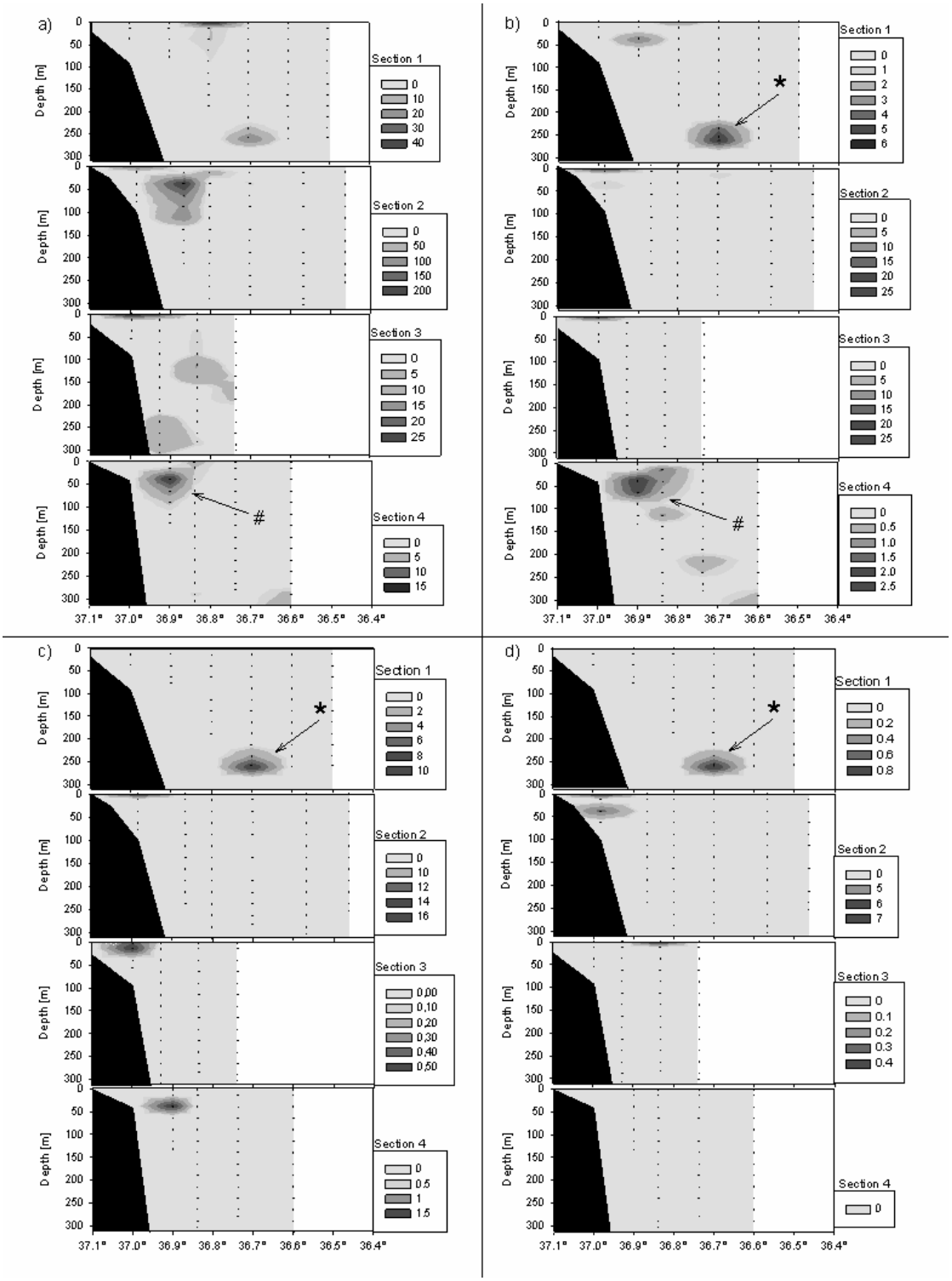

Figure 5.2: *Monodaeus couchi*. Contour plot of concentration (ind. 100 m^3) for zoea I (a), zoea 2 (b), zoea III (c) and zoea IV (d) encountered off the Algarve coast in 2006. The black shape represents sea-bottom, the black dots sampling location. "*" and "#" represent samples where zoeae II, III and IV, and zoeae I and II, were simultaneously present. In each quadrant, the four sections represent transect along the longitude 7.43W, 7.53W, 7.63W and 7.73W from top to bottom, respectively.

5.4 Results

5.4.1 Vertical distribution

Overall the abundance of *M. couchi* was much higher in 2006 than in 2007. In addition, patches of larvae composed of different development stages were observed in 2006 (Fig. 2 marked * and #). As expected, larvae abundance decreased as stage increased. In 2006, a significant interaction among larval stage, phase of day and depth was detected (Pseudo F= 2.16, df= 15, $p<0.005$; Figure 3). The abundance of zoea I during the day decreased with increasing depth, with higher catches at 0-50m and at 50-100 m (post-hoc tests, $p<0.048$ in all comparisons). At these depths, abundances were higher during the day than at night (post-hoc tests, $p<0.004$ in all comparisons). By contrast, zoea I abundance at night tended to increase with depth, but the differences were not statistically significant. In zoea II, abundances displayed a similar patterns as zoea I, but the differences between day and night catches were significant only at 0-50m depth interval (post-hoc test, $p<0.028$). Finally, zoea III and IV abundances also appeared to be higher during the day at the surface while at night abundances were higher below 100m. These results were however not significant, probably due to the very low numbers of zoea III and IV (overall abundances ± SE of 0.0638 ± 0.0600 ind.·100m^{-3}, 0.0029 ± 0.00006 ind.·100m^{-3}, 0.0019 ± 0.00009 ind.·100m^{-3} and 0.0012 ± 0.00007 ind.·100m^{-3} for zoeae I, II, III and IV, respectively). In 2007, abundances were significantly higher during the day than at night for all stages and depths (Pseudo F= 3.91, df= 1, $p<0.03$; Figure 4). Those results must however be interpreted with caution since larval abundances were extremely low (overall abundances of 0.0327 ± 0.0151 ind.·100m^{-3}, 0.0009 ± 0.00002 ind.·100m^{-3}, 0.0003 ± 0.00001 ind.·100m^{-3} and 0.0034 ± 0.0010 ind.·100m^{-3} for zoeae I, II, III and IV, respectively) and most larvae of the later stages (93% abundance of ZIII and IV) were encountered during the day at a single sampling site.

Finally, mean depth of larvae varied between years but overall the first stage was encountered at lower depth than later stages, with zoea IV average depth being the

deepest (average average depth of distribution for both years combined were 85.7, 119.6, 109.7 and 129.5 m for zoeae I to IV).

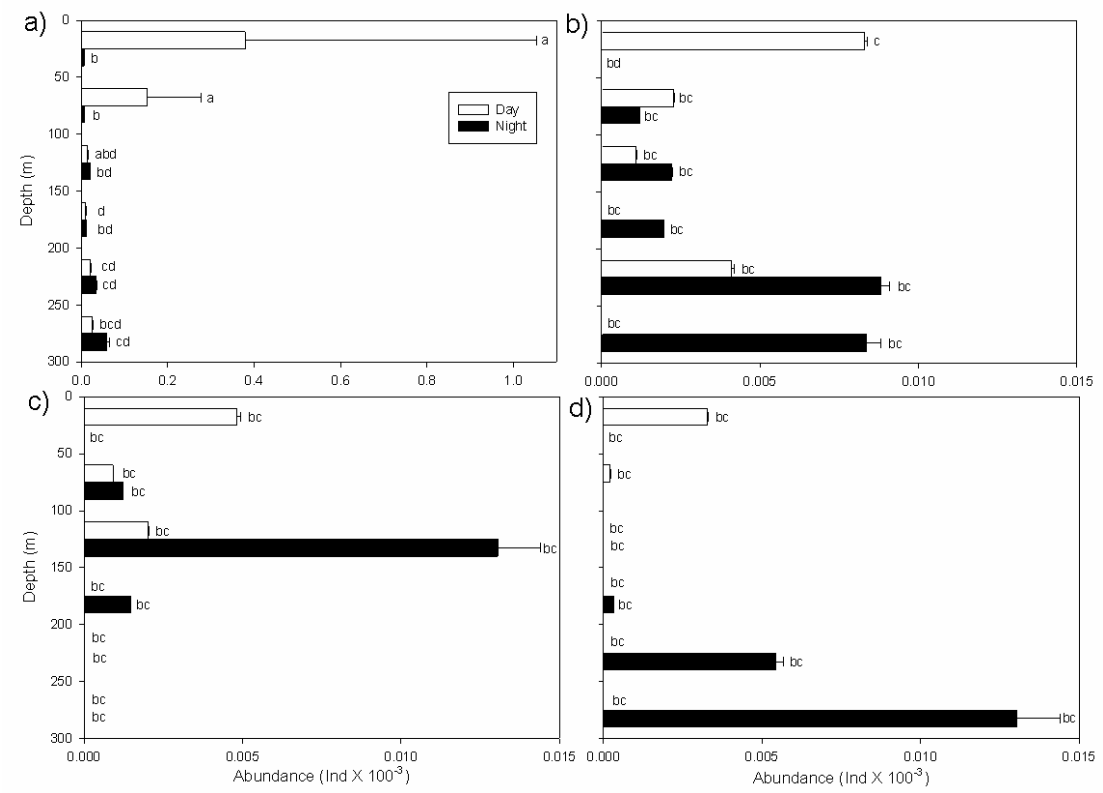

Figure 5.3: *Monodaeus couchi*. Abundance of larvae (ind. 100 m^3± standard error) with respect to depth for a) zoea I, b) zoea II, c) zoea III, and d) zoea IV as a function of the phase of the day for the 2006 cruise. White and black bars represend day and night samples, respectively. Identical letters above bars represent statistically similar abundances acording to post-hoc tests. The scale of abundance is different between zoea I and zoea II, III and IV.

5.4.2 Correlations among larval stages, and between larval stages and environmental parameters

Spearman correlation (rs) values between the concentrations of the different zoeal stages indicated that the abundance of zoeae I and II (rs=0.37), zoeae I and IV (rs= 0.30), and zoeae II and IV ($r_{s=}$ 0.45) were correlated in 2006. In 2007 only the concentrations of zoeae I and II were correlated ($r_{s=}$ 0.75; all values significant at α 0.05 probability level after Bonferroni correction, in 2006 and 2007). The difference in the results between years might be explained by the scarcity of zoeae III and IV.

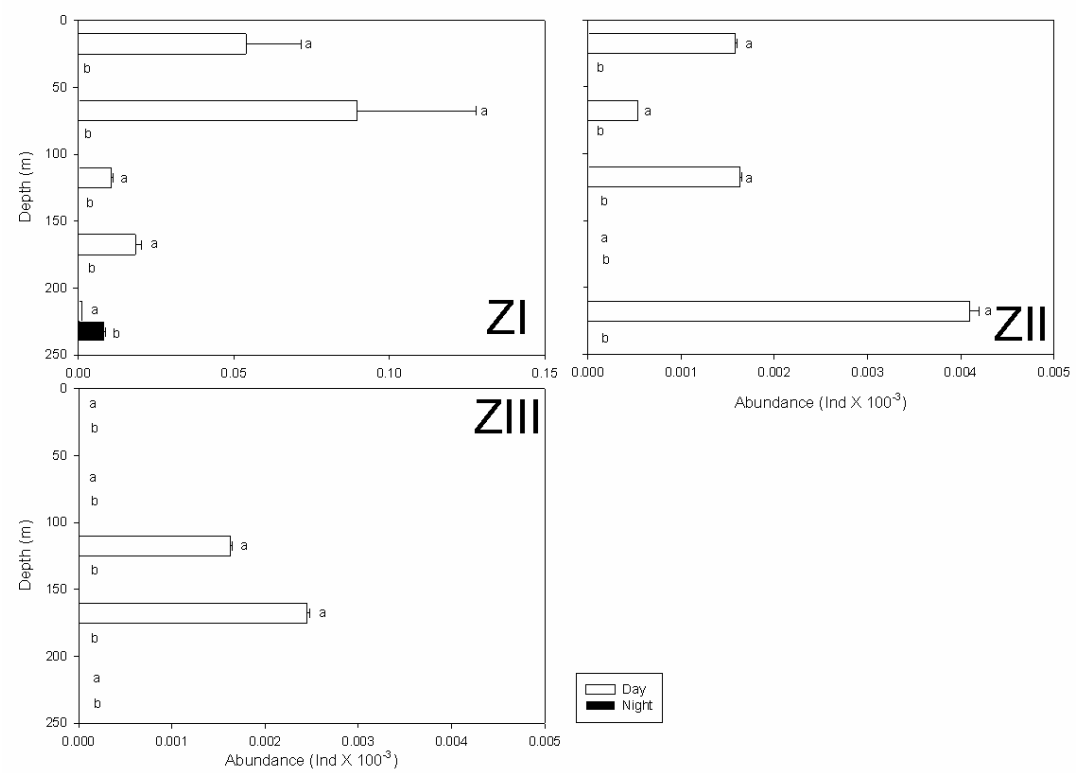

Figure 5.4: *Monodaeus couchi*. Abundance of larvae (ind. 100 m^3± standard error) with respect to depth for a) zoea I, b) zoea II, and c) zoea III as a function of the phase of the day for the 2007 cruise. White and black bars represend day and night samples, respectively. Identical letters above bars represent statistically similar abundances acording to post-hoc tests. The scale of abundance is different between zoea I and zoea II, III and IV.

In 2007 chlorophyll *a* levels had subsurface maxima between 30 and 70 m, decreased rapidly to about 180 m to become constant below that depth (Fig. 5). Temperature also decreased with depth and there was a sharp drop in temperature between 120 and 130 m indicative of a thermocline. The same pattern was observed for salinity, with a halocline located at the same depth range. Spearman correlations between environmental factors and the concentrations of larvae indicated that zoea I concentrations were positively correlated with chlorophyll *a* ($r_{s=}$ 0.27), salinity ($r_{s=}$0.23) and temperature ($r_{s=}$ 0.24; all values significant at $\alpha\ 0.05$ probability level after Bonferroni correction). In contrast, concentrations of zoea II were only positively correlated with levels of chlorophyll *a* ($r_{s=}$ 0,24). Zoeae III and IV were not correlated with any measured parameter. Catches of zoea I and II were maximal when chlorophyll *a* was above 0.3 mg m^{-3} (Fig. 5).

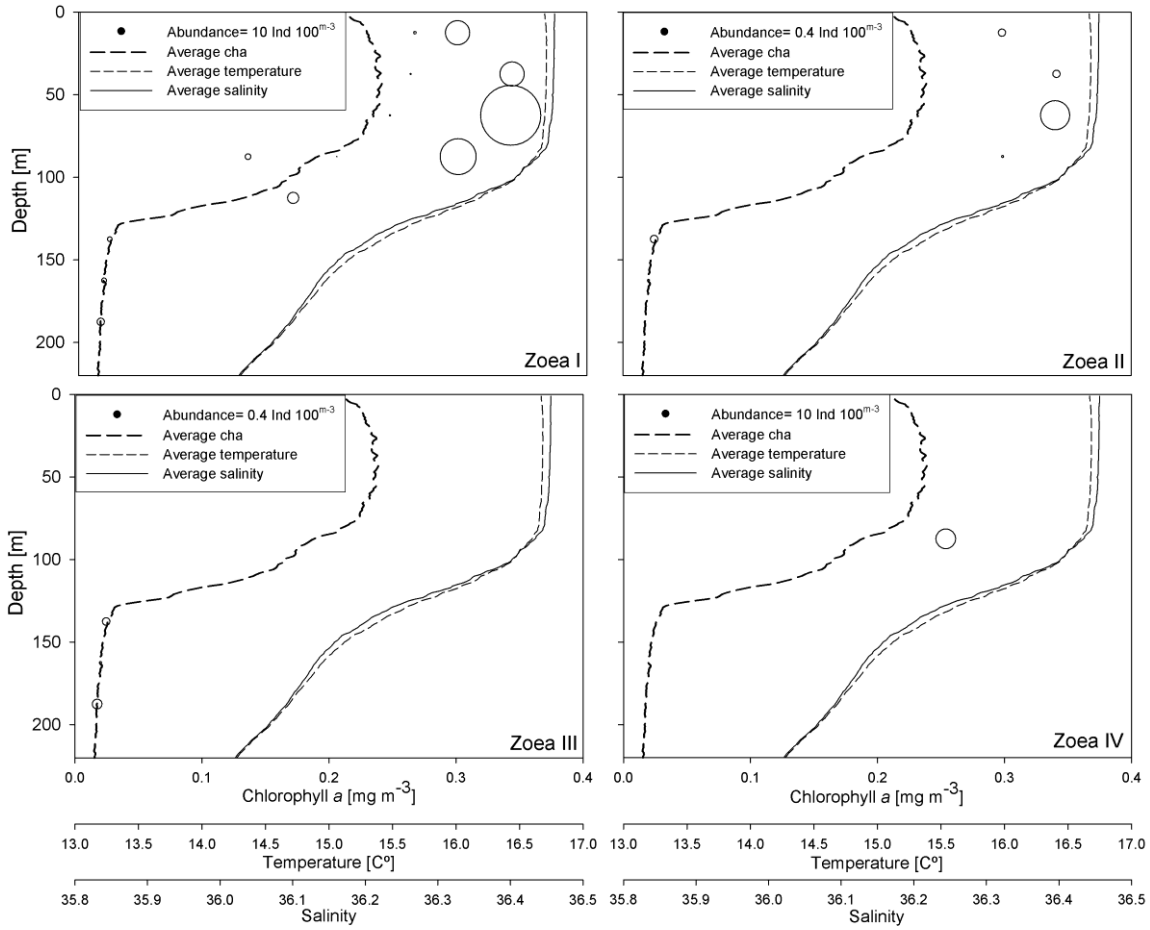

Figure 5.5: *Monodaeus couchi*. Standardized catch of zoea plotted as a function of depth (m) and chlorophyll *a* concentration (mg m^{-3}) during the 2007 campaing off the coast of Algarve. The lines represents the average profile of chlorophyll *a*, temperature (ºC) and salinity concentration calculated from measurements made at all the stations.

5.5 Discussion

In this study, the coast off southern Portugal was sampled for two consecutive years. Overall, larval abundance of *Monodaeus couchi* in 2006 was higher than in 2007, all larval stages were found at depths above 300 m with the early stages concentrated in chlorophyll-rich layers, and the differences in abundance according to phase of day and depth indicate the occurrence of reverse diel vertical migration.

The occurrence of *Monodaeus couchi* larvae in the surface layer indicates that larvae hatching from benthic females from the slope and mud volcanoes populations are

able to rapidly ascend to the surface, crossing the thermocline around 120 m, to feed in surface waters, as shown by the positive correlation between first stages and chlorophyll *a*. This migration to the surface is widespread among first stage crab zoeae and results from negative geotaxis and high barokinesis (Ott and Forward 1976; Schembri 1982; Sulkin 1973). Crab larvae are able to cross sharp picnoclynes during migration towards surface waters, and this has also been demonstrated in early zoeal stages of *Geryon quinquedens* (Kelly et al. 1982), another deep-water brachyuran. Average larval depth increased with larval stage, also in line with numerous reports showing a preponderance of positive geotaxis in older larvae (Sulkin 1973), especially at the transition to the megalopa stage that must settle to appropriate benthic habitats. In the case of *M. couchi,* megalopae will have to settle several hundred meters deeper on the seabed, which, together with the attrition of densities with age, would explain the absence of megalopae in our samples.

In zooplankton, diel vertical migration (DVM) behaviors vary interspecifically (Han and Straskraba 2001). Species can undergo any of the three described types of migration or not migrate at all (Forward 1988): 1) nocturnal diel migration, the most common form where larvae rise to surface water during the night to feed and sink below the photic zone during the day, 2) reverse diel vertical migration where larvae rise to the surface during the day and sink to deeper water at night and 3) twilight migration where larvae undergo two upward migrations near sunset and sunrise. Even intraspecifically, DVM behavior might vary with time, location (*e.g.* distance from coast) or environmental conditions such as fluctuations in predator or prey abundances or vertical stratification (Irigoien et al. 2004; Osgood and Frost 2004). In the present study, *M. couchi* was most abundant during the day in the top hundred meter of the water column, while at night abundance increased with depth. In the Algarve area *M. couchi* thus underwent reverse diel vertical migration which was most defined in the 2006 sampling period, probably due to the overall higher larval abundances observed.

In several studies, reverse DVM has been suggested as a defense against predators that also migrate vertically. As observed in various copepod species, when predators exhibit nocturnal DVM, their prey may undergo reverse DVM (Irigoien et al. 2004; Osgood and Frost 2004). This behavior has been associated with areas where the predators rely more on tactile stimuli than sight to capture prey, as is the case for many invertebrate predators (e. g. chaetognaths and gelatinous zooplankton) which themselves are undergoing regular DVM as a way to escape fish predators (Tester et al. 2004). Reverse DVM has also been observed in areas with lower abundances of predatory fish (Lagergren et al. 2008). Since shifts in composition and abundance of predator populations occur, the prey must be able to adjust their behavior accordingly and, therefore, temporal and geographical plasticity in DVM behavior of *M. couchi* larvae could exist in response to changing environmental conditions.

The abundance of zoea I and II was found to be positively correlated with chlorophyll *a* levels. There are two mechanisms which might explain the correlation between the two factors. First, *M. couchi* larvae, their prey and phytoplankton might be passively aggregated together due to hydrodynamic processes. Second, larvae responding to physical or biological factors might actively swim to avoid unsuitable environments (Folt and Burns 1999). In contrast, zoeae III and IV abundances were not correlated with chlorophyll *a*, which is explained by the highly variable depth distribution as well as very low abundances of these stages. Those larger larvae might shift their diet to different, larger prey items that might be distributed more evenly in the water column resulting in the observed pattern.

Our results indicate that newly hatched larvae of *Monodaeus couchi*, and probably of other open ocean benthic decapods, can migrate hundreds, if not thousands, of meters to the photic layers of the water column where food is more abundant. Late-stage larvae were encountered deeper, initiating the return migration to the sea bed. Following the first ontogenetic migration to surface layers, diel vertical migration behavior of such species is relatively unknown. *M. couchi* was found to perform a

reverse migration within the top 300 m of the ocean, but variability might exist in DVM behavior depending on the species, location and environmental conditions.

Acknowledgments

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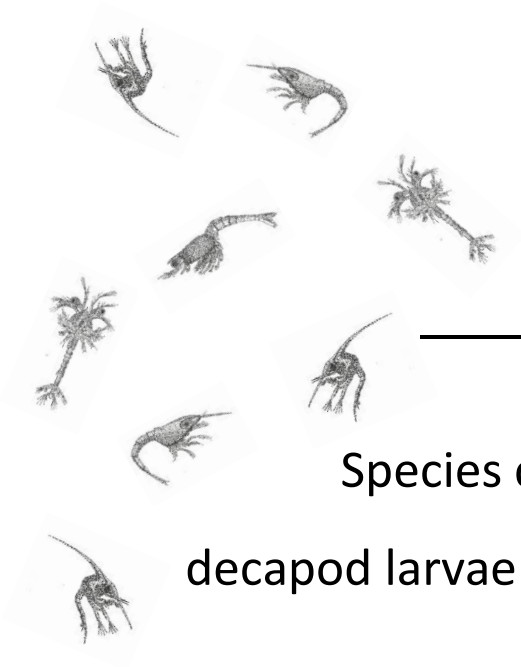

Chapter 6

Species composition and distribution of decapod larvae off the South Coast of Portugal

In review; Journal of Sea Research

Chapter 6

Species composition and distribution of decapod larvae off the South Coast of Portugal

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Keywords: Crustaceans, Invertebrate Larvae, Vertical Distribution, Iberian Peninsula, south and southwest coast of Portugal.

6.1 Abstract

Larval stage is critical in the crustacean life cycle as they often represent the only dispersal phase. Because of their reduced swimming ability, circulation pattern is the primary factor influencing dispersal. Knowing larval distribution and abundance is necessary to predict location and size of the breeding population. In this study, spatial distribution and abundance of the decapod larvae were assessed. Two week-long campaigns were conducted at the end of January in 2006 and 2007, respectively sampling 28 and 47 sites in three different regions off the South Coast of Portugal; the coast of Algarve, Sagres and Alentejo. Larvae were collected by oblique plankton hauls from the surface to 200-300 m. In addition to *in* situ measurements of environmental parameters, an oceanographic model was used to better understand the processes affecting larval dispersal. Both years, *Monodaeus couchi* was the most abundant species, followed by *Goneplax rhomboides*. However, coastal species such as *Atelecyclus rotundatus*, *Pilumnus* sp., *Processa* spp. and *Philocheras* spp. that dominate in the 2006 campaign, were almost completely absent in the 2007, more offshore, campaign. Offshore decapod composition off the Algarve coast was similar between years even though overall decapod catch in 2007 was much lower than in 2006. Larval distribution and abundance varied between sampling area which is likely to be the result of differences in hydrographic conditions and adult distribution. Indeed, an upwelling event was occurring off the southwest coast of Portugal at the time of sampling, which separated the Northeastern part of Alentejo from the Southwestern part which had lower decapod abundances and diversity than it Northeastern counterpart. Additionally, the Northeastern part of the Alentejo area was dominated by the crabs *Cancer* spp., *Carcinus maenas* and *Atelecyclus rotundatus* which were completely absent from the Southwest. In the NE, the most abundant species were retained onshore while offshore transport occurred in zooplankton and to a lesser extent in some shelf species.

6.2 Introduction

Larval dispersal and supply are recognized as fundamental mechanisms regulating the dynamics of marine populations (Caley and Carr 1996; Kritzer and Sale 2006) and therefore they are key to the management of fisheries resources, the understanding of the spread of invasive species, the design of marine protected areas and the assessment of climate-change effects (Levin 2006; Paulay and Meyer 2006). In benthic decapod crustaceans the larval phase is a critical stage as it often represents the principal agent of connectivity among local populations and the only period available for dispersal. Decapod larvae generally lack strong horizontal swimming abilities but are capable of regulating their position in the water column through vertical swimming. A large body of evidence (reviewed by Queiroga and Blanton 2005) shows that decapod larvae respond to environmental and internal stimuli to trigger or inhibit vertical swimming. Changes in depth distribution related to those cues will influence directly or indirectly growth, feeding, and mortality, settlement to benthic habitats and, ultimately, dispersal and transport.

During the period from hatching to settlement, mortality is high and only larvae that remain in suitable environments will be able to thrive. General circulation pattern is the main primary factor influencing larval transport on a large spatial scale (>1000 km). Eddies, estuarine plumes or even upwelling event, affect larval distribution on smaller scales (e.g. Queiroga et al. 2007). Finally, on a short scale (<100 m) larvae can regulate their position and respond to changes in the water properties such as light, temperature, salinity, hydrostatic pressure (Epifanio and Garvine 2001; Folt and Burns 1999; Forward 1988), and biotic factors (*e.g.* phytoplankton abundance, predator and prey concentration), thus actively influencing their horizontal or vertical position. Concerning biotic factors, state of feeding influence vertical distribution and predation is one of the largest cause of mortality (McConaugha 1992). Presence of appropriate food items, not only in terms of size, catchability and energetic value, but also of good biochemical composition, will further promote or hinder successful development (Fuchs and Franks 2010). Additionally, variations in physical factors such as

temperature and salinity will affect duration of the larval phase which could impact survival and successful settlement (Anger 2001).

Because of the high diversity of habitats colonized by decapods, larval developmental strategies vary greatly. Benthic species inhabiting coastal and estuarine areas as adults, but which develop in the open ocean during the larval phase, possess mechanisms that allow them to concentrate inshore before settlement to appropriate adult habitat takes place (dos Santos et al. 2007; Morgan et al. 2009; Queiroga and Blanton 2005). In contrast, adults of offshore decapods can be either pelagic (*e.g.* Sergestids shrimps), benthopelagic (many Caridean shrimps) or benthic (many brachyuran and anomuran crabs, as well as lobsters). Post-larvae of offshore benthic decapods will often require settling in substrate suitable for juvenile and adult development and survival. In contrast, since in pelagic species the larval stage is not the only stage available for dispersal, it can be assumed that metamorphosis is independent of substrate conditions, being controlled only by physical factors and physiological state.

In either case, circulation patterns will have a large impact on larval dispersal. Large scale circulation pattern generally have a marked seasonal pattern; however variations can be observed over the period of day to weeks. A good understanding of the hydrography of the sampling area is therefore critical to better understand larval distribution. Off the South and Southwest coasts of Portugal, our area of study, ocean circulation has a marked seasonal pattern, related to atmospheric forcing and subjected to the influence of the large scale circulation (Azores Current Branch; reviewed by Peliz et al. 2005; Relvas et al. 2007). During summer, circulation along the South-West coast of Portugal (Alentejo coast, see Fig. 1) is characterized by upwelling and its associated southward flow that brings up colder water near the coast and transports surface waters offshore. Poleward flow is typically observed during winter, although upwelling events are frequently reported during this season (Relvas et al. 2007). The waters that feed this poleward flow during winter have hydrographic characteristics (salty and warm) similar to those found in the Northern branches of the

Azores current. Southerly winds along the West coast further reinforce the poleward flow thus promoting coastward transport (Frouin et al. 1990).

Surface circulation in the Gulf of Cadiz is cyclonic, with westward circulation in the Algarve region (Fig. 1) along the deeper slope (600 m to 1300 m depth) related to the Mediterranean Underflow (MU). The presence of a flow in the eastward direction (called Gulf of Cadiz Slope Current), centered above isobaths 200m and feeding the Mediterranean basin with Atlantic surface waters through the strait of Gibraltar (Fig. 1), is also observed along the upper slope of the Algarve coast. The mean circulation of the Gulf of Cadiz is studied in detail in Peliz et al. (2007).

Despite the high number of studies investigating decapod larvae abundances in coastal areas (DiBacco et al. 2001; Queiroga et al. 2006; Storm and Pedersen 2003), such studies remain scarce in offshore areas and, more specifically, very little is known about decapod larvae abundances in offshore waters of the Iberian Peninsula (dos Santos and Peliz 2005)since most studies have focused on coastal areas, *i.e.* areas over the continental shelf with a bottom depth lower than 200 m (deCastro et al. 2003; dos Santos et al. 2008; Gonzalez-Gordillo and Rodriguez 2003; Paula 1987; Queiroga 1996).

The main objective of the present study was to obtain data on the composition, abundance and distribution of decapod crustacean larvae off the south Portuguese coast during winter of the years 2006 and 2007. Attention was focused on annual and regional differences and onshore-offshore contrasts. Due to the occurrence of an upwelling event off the SW coast of Portugal during the 2007 sampling period, we used an oceanographic numerical model to explain the formation of the upwelling front and provide an interpretation of the physical transport mechanisms that underlie larval distribution and drift in this area. The outputs of the model helped elucidate the mechanism generating the segregation of the assemblages in the Alentejo region in 2007 found on either side of the front. Stronger knowledge of variations in larval dispersal and the processes causing them will ultimately help us understand better how global changes might impact the lifecycle of decapod species.

6.3 Material and Methods

6.3.1 Field collection

In order to investigate the distribution of Decapod larvae, two oceanographic cruises were conducted off the south coast of Portugal, one from January 29th to February 5th of 2006 and the other from January 24th to 31st of 2007.

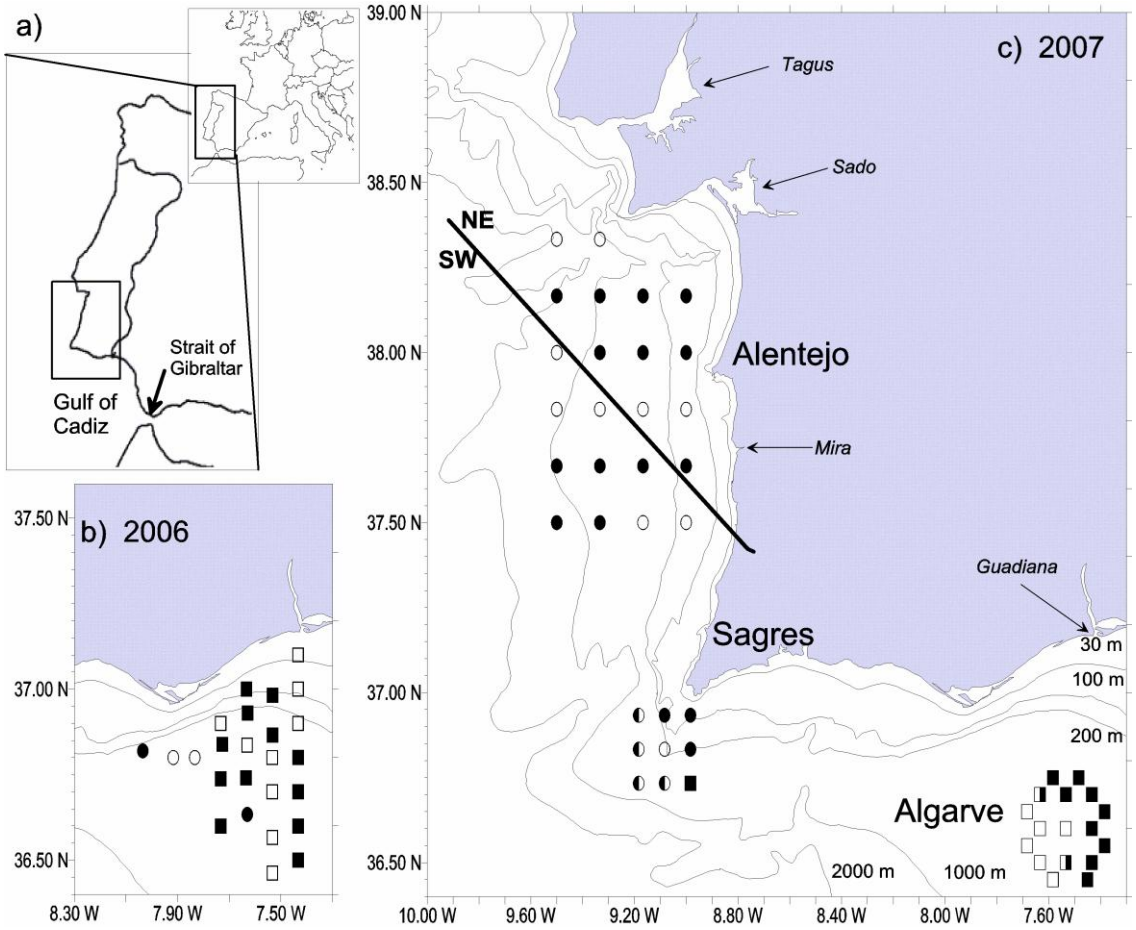


Figure 6.1: . *Sampling area*: Map showing the Iberian Peninsula (a) and the locations of sampling sites in 2006 (b) and 2007 (c). In 2006 (b) sampling started on January 29th at 12:00 and ended on February 1st at 16:30. In 2007 (c) sampling started at station 1 on January 24th at 15:00 and ended January 31st at 11:15. For both years, circles and squares represent sampling with LHPR and Bongo, respectively. Black and white indicate night and day sampling, respectively, while half colored represent two independent sampling taken during the day and at night. The Alentejo area was divided, as indicated by the black line, into a northeast and a southwest subregions based on oceanographic and modelling results. Arrows indicate the major rivers named in italic.

In 2006, a total of 25 locations were sampled off the south coast of Algarve, between Cape St. Maria and the Spanish border (Fig. 1b). All locations in this region were sampled by oblique hauls using a Longhurst-Hardy Plankton Recorder (LHPR) equipped with a 42 cm diameter cone, 280 μm mesh net and a flow meter to sample 21 locations (9 day, 12 night). The water column was resolved into approximately 25 m depth strata, from the surface to 10 m above the bottom, or to a maximum depth of 300 m at the deeper locations. Due to technical problems with the LHPR, 4 additional locations (2 day, 2 night) were sampled with a Bongo net equipped with a 335 μm mesh net and a flow meter from the surface to 200 m. Technical problems precluded the collection of hydrographic measurements during the 2006 cruise.

Samples in 2007 were taken in 3 different regions (Fig. 1c). Again, due to technical problems, the sampling was partially made with a Bongo net in replacement of the LHPR. A total of 17 locations were sampled off the south coast of Algarve between Cape of St. Maria and the Spanish border, between January 24th and 27th. From these, 9 locations were sampled twice, at approximately 24 h intervals. The remaining 8 were only sampled once. Overall, 14 samples were taken during the day and 10 at night. In this area, all locations were sampled with LHPR from the surface to 200 m. Sampling in this region differed from that conducted in 2006 because all locations sampled were off the shelf (bottom depth >200m). In the second area, located off Cape of Sagres at the western tip of the Algarve coast, 9 locations were sampled twice, with 6 samples collected during the day and 12 at night, between January 27th and 29th. The 5 most coastal locations were on the continental shelf and will hereafter be referred to as “coastal” (bottom depth < 200m). Finally 22 locations were sampled off the coast of Alentejo, 9 during the day and 13 at night, between January 29th and 31st. From those sampling sites, 4 were located on the continental shelf. Again due to technical problems affecting the LHPR, the Sagres and the Alentejo regions, (except for one location off of Sagres) were sampled with the Bongo net from the surface to 10 m above the bottom, or to a maximum depth of 200 m. Overall, 29 samples were taken during the day and 35 during the night. For both oceanographic cruises, the organisms sampled were immediately fixed in 4% buffered formaldehyde and brought

to the laboratory for taxonomic identification. Decapod larvae were identified to the lowest taxonomic level possible and staged, according to dos Santos and Gonzalez-Gordillo (2004) and dos Santos and Lindley (2001). Additionally, the zooplankton biovolume at each station was measured and used as a proxy for zooplankton biomass.

In 2007, measurements of vertical distribution of temperature and salinity were made with a Seabird SBE 9 plus CTD, which was deployed at each location immediately after collection of the plankton samples. Concerning atmospheric forcing, the wind field for the Alentejo region was extracted from QuikSCAT for the month of January 2007 (average between 9.5-9.0W and 37.25-37.75N). Data were extracted from CERSAT reanalysis data (IFREMER, France; cersat.ifremer.fr).

6.3.2 Statistical analysis

Bongo and LHPR nets have different efficiencies. Stehle et al (2007) demonstrated that estimates of concentrations produced from depth-integrated oblique hauls by the LHPR were up to 7 times higher, depending on *taxa*, than those obtained by the Bongo net. This effect seems to be related to escape from the slow-towed Bongo net. Therefore, in order to compare the abundance of Decapod larvae caught with the LHPR and the Bongo nets, the catch values of the latter were divided by a ratio estimator of 0.45, as was obtained for Decapod zoeae from both day and night samples of the coast of Portugal. This conversion factor was found to produce a maximum error of 37% within a 95% confidence level. Similarly, zooplankton biovolumes were adjusted by a conversion factor of 0.12, as obtained for total biomass. This conversion factor was found to produce a maximum error of 4% within a 95% confidence level (Stehle et al. 2007). This is a sub-optimal design because the correction factor may not account for *taxa*-specific differences in efficiency between the two nets. However, on average the actual error will be much less than 37% and, as the most abundant *taxa* have similar sizes, we might expect small differences in net efficiencies across *taxa*. Additionally, most of the decapod species sampled in the study by Stehle et al. (2007) were the same as collected in the present study. We

therefore believe that the use of this correcting factor to compare results obtained by the two nets is justified. In all samples, the number of individuals caught was expressed in number of larvae per 100 m^3.

In order to test for differences in decapod larvae species composition and abundance between years and sampling locations, three distinct analyses were performed with PRIMER (Plymouth Routines In Multivariate Ecological Research) 6.1 software equipped with PERMANOVA + 1.0. First, the differences between coastal and offshore location were evaluated for the 2006 samples in the Algarve. The separation between the coastal and offshore region was made following the 200 m isobath. Then, geographical differences were assessed using the 2007 data. In the case of the Alentejo sampling, between January 29th and 31st 2007, direct inspection of hydrographic fields (salinity and temperature), indicated that two different water masses were present, separated by a front, visible in the salinity and temperature distribution (Fig. 4), and also inferred from the corresponding modeled fields described below (Fig. 6). As a result, this region was divided into two sub-regions, Northeast and Southwest that were separated by the front, and sampling sites were therefore classified into four groups in 2007, Algarve, Sagres, Alentejo Southwest and Alentejo Northeast (Fig. 1). Finally, inter-annual differences were assessed between the 2006 offshore sampling sites and the Algarve region of 2007. Additionally, the effect of the day/night cycle on decapod abundance was factored in each analysis. In all cases, data were square root transformed prior to analysis and a resemblance matrix was created using Bray-Curtis similarities. Significant differences were tested using PERMANOVA. In order to visualize similarities and dissimilarities of the data, a Principal Coordinate Analysis (PCO) was performed. The contribution of the main two axes was plotted and a vector overlay was added to indicate the strength of the relationship between each species and the PCO axes using Pearson correlation. Only species with a vector length greater than 0.5 were plotted.

6.3.3 Oceanographic model

Inspection of the temperature and salinity fields obtained in the 2007 cruise indicated the occurrence of a pronounced frontal structure off the Alentejo coast (see Results). In order to elucidate the process originating the frontal structure, which takes place over an area much larger that the region sampled, as well as its influence on the observed larval distribution during the 2007 cruise, numerical model simulations of the ocean circulation were conducted using the primitive equation hydrostatic Regional Ocean Modeling System (ROMS; Shchepetkin and McWilliams 2003). The present configuration, represents an improvement and extension of those used by Peliz et al. (2007) and Oliveira et al (2009). In the scope of the present work, a summarized description is given for the present configuration. For full details about the model configuration the reader is referred to the above works. The main domain of the model includes the Western Iberian margin, from the Gulf of Cadiz (34.5 ºN) to the Bay of Biscay 45.5 ºN, and from 12.5 ºW to the Strait of Gibraltar 5.5 ºW. Boundary conditions were taken from a larger domain in such a way that it allowed solving the influence of the large-scale circulation (Azores current) on the main domain, as well as the influence of the Mediterranean outflow through Strait of Gibraltar. The model was run for the period 2001-2008 and was forced by the NCEP2 air-sea fluxes (www.ncep.noaa.gov), QuikScat reanalyzed satellite winds extracted from CERSAT (cersat.ifremer.fr) and river input.

6.4 Results

6.4.1 Decapod larval composition and abundance in 2006 and 2007

The list of decapod species or genera for which larvae were collected is given in Table 1. From the 49 *taxa* sampled, 30 were encountered in both years, 7 in 2006 only and 12 in 2007 only. Most *taxa* were present at low densities (<0.05 ind $100m^{-3}$). Overall, larval abundance was much higher in 2006 than in 2007 (Fig. 2a). Abundance was

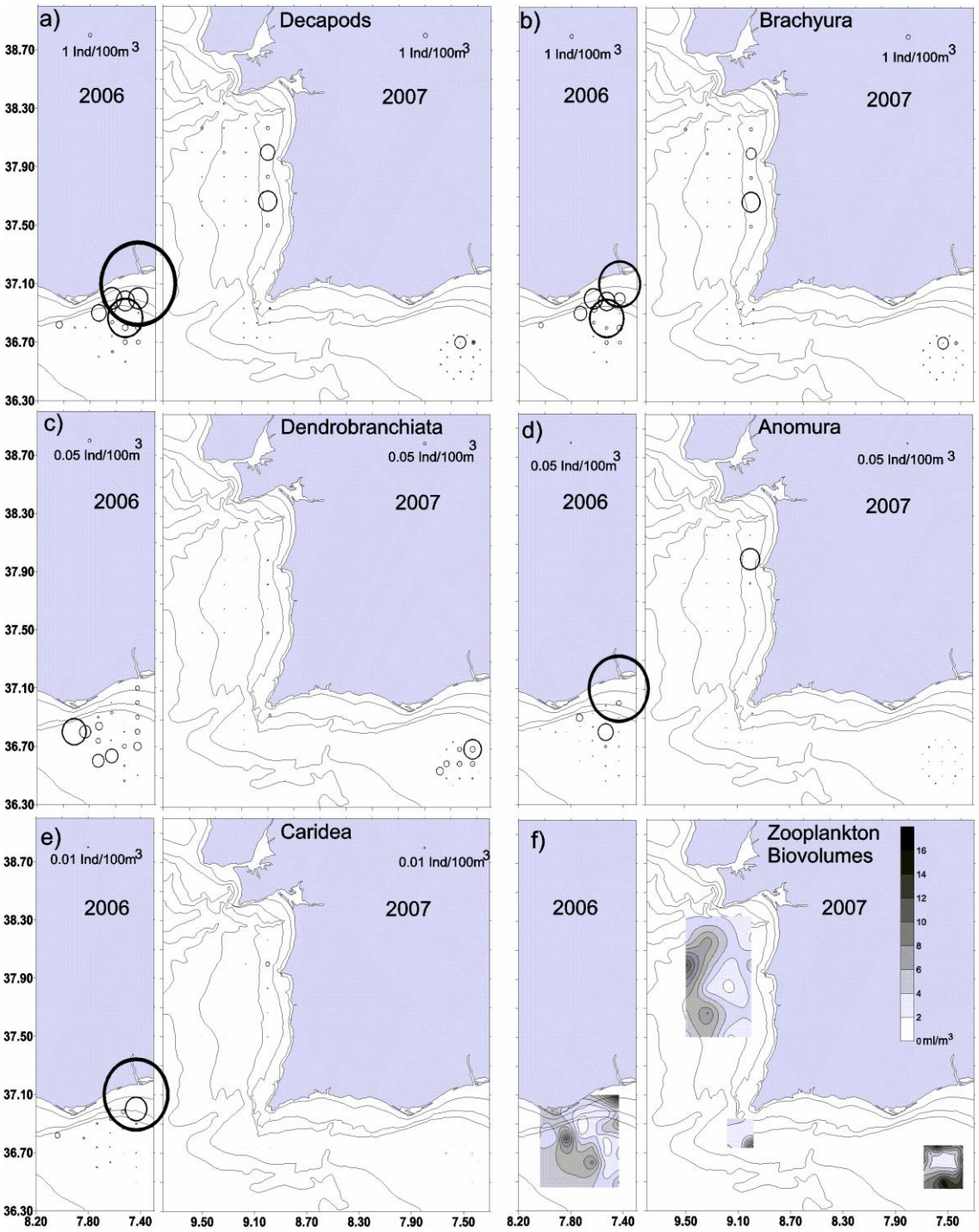


Figure 6.2: *Decapod abundances:* Vertically integrated concentrations of decapod larvae in locations sampled in 2006 and 2007, for (a) all Decapoda, (b) Brachyura, (c) Dendrobranchiata, (d) Anomura, (e) Caridea larvae and (f) zooplankton biovolumes. In a) to e) the size of the circle represents larval concentration according to the scale shown in each panel (ind 100 m^{-3}). In f) the color scale represents the abundance of zooplankton (ml m^{-3}).

highest close to the coast. In 2007 high larval abundance was also encountered off the coast of Alentejo in the coastal side of the front (Fig. 1). In both years, brachyuran crabs were the most common group, representing 70% and 77% of total Decapoda abundance for 2006 and 2007, respectively. They were mainly *Monodaeus couchi*, *Goneplax rhomboides*, *Pilumnus* spp*., Atelecyclus rotundatus* and *Liocarcinus* spp. Consequently, brachyuran larvae were also highly abundant in 2006 close to the Guadiana and Mira rivers (Fig. 2b). The second most abundant group was the dendrobranchiata shrimps (13% and 18% total abundance, respectively) in which the most common species were *Solenocera membranacea* and *Sergestes* spp.. Their abundance was very low close to the coast and predominated in the Algarve as opposed to the Sagres and Alentejo regions (Fig. 2c). Hermit crabs represented 9% and 3% of total abundance in 2006 and 2007 respectively (Fig. 2d). The most common were *Anapagurus* spp. and *Pagurus* spp. (both years) and the squat lobster *Munida* spp. (in 2007). Finally, caridean shrimps made up 8% and 2% of total abundance in 2006 and 2007 respectively (Fig. 2e), with *Alpheus glaber*, *Processa* spp. and *Philocheras* spp. being very abundant in 2006. In comparison, caridean shrimps abundance was very low in 2007. Their abundance was high only in the Northeast of the Algarve sampling area, which is influenced by the river Guadiana. Numbers were very low in the offshore sampling sites. The other groups (Gebiidea, Astacidea and Achelata) represented together less than 1% of the larvae collected in both years. In 2007, biovolumes, representative of overall zooplankton abundance, were much higher offshore than onshore in the Alentejo area (Fig 2f).

Table 6.1 (next page): Mean abundance (±SD, ind. 100 m–3) of decapod larvae from the most abundant taxa collected during the 2006 and 2007 campaigns. A total of 25 locations were sampled in 2006 off the coast of Algarve and 66 in 2007 off the coasts of Algarve, Sagres and Alentejo. Area indicates whether the taxa occurred in shelf (s) or oceanic (o) waters of the Algarve region in 2006. Region indicates whether the taxa occurred in the Algarve (Alg), Sagres (Sag) SW Alentejo (ASW) or NE Alentejo (ANE) regions in 2007.

Taxa	2006		Area	2007		Region
Brachyura						
Monodaeus couchi	0.544±	1.381	co	0.059±	0.357	Alg/Sag/ASW/ANE
Goneplax rhomboides	0.299±	0.546	co	0.010±	0.047	Alg/Sag/ANE
Pilumnus sp.	0.188±	0.483	co	0.0004±	0.0013	Sag/ASW/ANE
Atelecyclus rotundatus	0.188±	0.680	co	0.018±	0.106	Sag/ASW/ANE
Liocarcinus spp.	0.140±	0.511	co	0.004±	0.016	Alg/Sag/ASW/ANE
Maja sp.	0.013±	0.049	co	0.0001±	0.0005	Alg
Nepinnotheres pinnotheres	0.005±	0.014	c	0.00001±	0.00011	Sag
Ebalia sp.	0.004±	0.012	co	0.0008±	0.0038	Alg/Sag/ASW/ANE
Polybius henslowii	0.003±	0.013	o			
Polybiinae n id	0.002±	0.006	o			
Geryon sp.	0.001±	0.005	c	0.0003±	0.0027	Alg/Sag
Xantho sp.	0.001±	0.003	c			
Cancer pagurus				0.0101±	0.0353	ANE
Carcinus maenas				0.0023±	0.0164	ANE
Macropipus sp.				0.0007±	0.0028	Alg/Sag/ASW/ANE
Eurynome sp.				0.0006±	0.0033	Alg/ANE
Parthenope sp.				0.0001±	0.0005	Alg
Asthenognathus atlanticus				0.00001±	0.00006	ANE
Rochinia carpenteri				0.000004±	0.000036	ANE
Anomoura						
Anapagurus sp.	0.069±	0.284	co	0.0025±	0.0159	Alg/Sag/ANE
Munida sp.	0.038±	0.137	co	0.0003±	0.0015	Alg/ANE
Pagurus sp	0.037±	0.135	co	0.0021±	0.0066	Alg/Sag/ASW/ANE
Diogenes pugilator	0.010±	0.033	c	0.0002±	0.0015	ASW/ANE
Galathea sp.	0.008±	0.032	c	0.0002±	0.0012	Sag/ANE
Pisidia longicornis	0.003±	0.014	c	0.0001±	0.0007	ANE
Nematopagurus longicornis				0.0002±	0.0013	Alg/Sag
Dendrobranchiata						
Solenocera membranacea	0.019±	0.027	co	0.001±	0.003	Alg/Sag/ASW/ANE
Sergestes sp.	0.018±	0.029	co	0.005±	0.019	Alg/Sag/ASW/ANE
Gennadas sp.	0.006±	0.012	co	0.0003±	0.0015	Alg/Sag/ASW/ANE
Sicyonia carinata	0.002±	0.009	o	0.000±	0.003	Alg
Sergia robusta	0.00002±	0.00009	co	0.008±	0.031	Alg/Sag/ASW/ANE
Benthesicymus sp.				0.0005±	0.0019	Alg/Sag/ASW
Parapenaeus longirostris				0.00013±	0.00070	Alg/ANE
Caridea						
Alpheus glaber	0.395±	1.800	co	0.0006±	0.0042	Sag/ASW/ANE
Processa sp.	0.121±	0.427	co	0.0006±	0.0029	Alg/Sag/ASW/ANE
Philocheras sp.	0.098±	0.366	co	0.0002±	0.0014	Sag/ANE
Eualus sp.	0.020±	0.069	co	0.0001±	0.0003	Sag/ASW/ANE
Athanas nitescens	0.013±	0.033	c	0.0000±	0.0002	Sag/ASW/ANE
Plesionika sp.	0.011±	0.021	co	0.0002±	0.0007	Sag/ASW/ANE
Crangon crangon	0.003±	0.016	c	0.0000±	0.0002	Sag
Nematocarcinus sp.	0.002±	0.005	co			
Acanthephyra sp.	0.001±	0.006	c	0.00007±	0.00053	Alg
Lysmata sp.	0.00002±	0.00008	c			
Palaemon sp.				0.0001±	0.0007	Sag/ANE
Other groups						
Nephrops norvegicus	0.001±	0.003	o	0.0004±	0.0020	Alg
Upogebia sp.	0.001±	0.004	o			
Palinurus sp.				0.0000±	0.0001	Sag/ASW/ANE
Scyllarus sp.	0.0001±	0.0004	o			

6.4.2 Patterns of decapod larval distribution in the Algarve region in 2006

Abundance of larvae in the Algarve region in 2006 was much higher in the coastal side of the front than offshore, mainly due to the high number of crabs and Caridean shrimp larvae encountered in coastal areas (Fig. 2a, b and e). A total of 9 taxa were found only over the shelf, 6 only offshore, and 20 both on the shelf and offshore (Table 1). Results of the PERMANOVA indicated that distribution and abundance varied between the coastal and offshore sampling sites in 2006 ($p<0.0001$) but there was no statistically significant difference in larval distribution between day and night samples ($p>0.064$). Coastal sites were dominated by larvae of the crabs *Goneplax rhomboides*, *Pilumnus* spp., *Atelecyclus rotundatus*, and *Liocarcinus* spp. and of the caridean shrimps *Plesionika*, spp., and *Philocheras* spp. In contrast, the most common species encountered offshore were *Monodaeus couchi* and *Sergestes* spp. (Fig. 3).

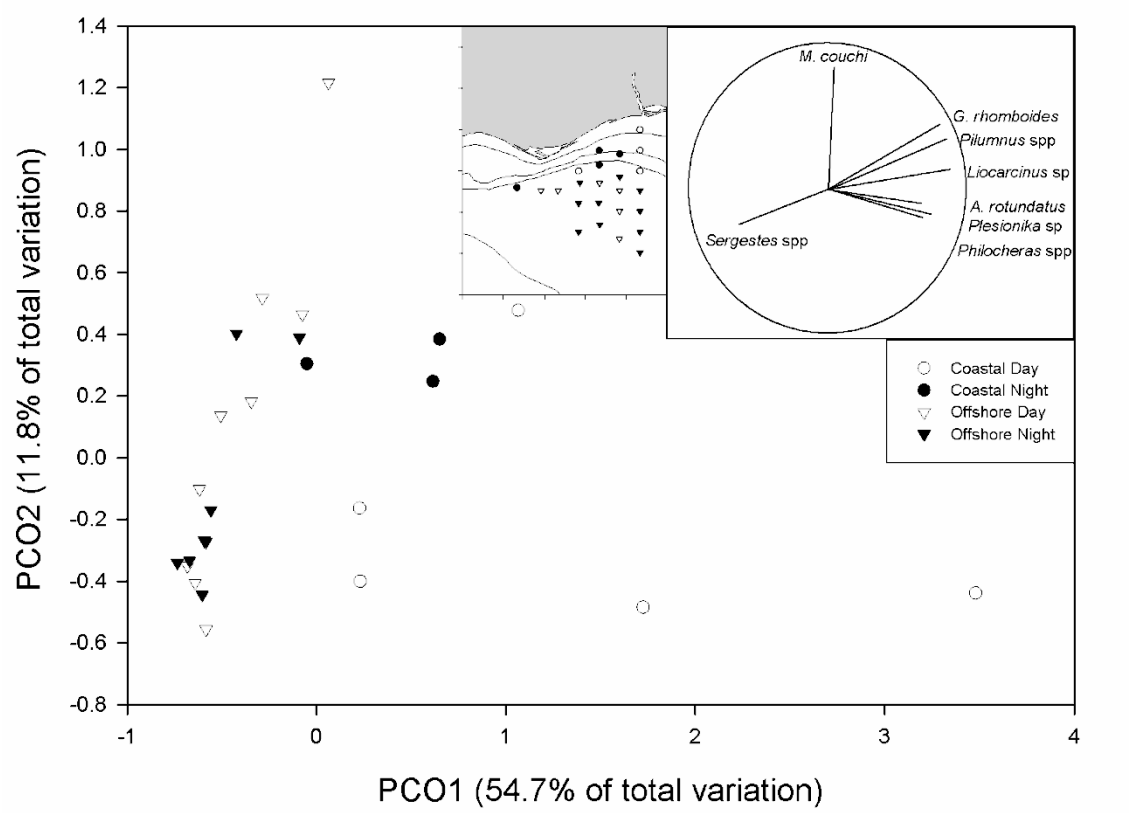


Figure 6.3: ***Principal Coordinate Analysis (PCO) comparing coastal vs. offshore decapod distribution in 2006*****. Inset map indicates sampling locations. Black and grey triangles represent coastal and offshore sampling site, respectively. Vector length and direction indicate strength and sign of the relationship between the variable and the PCO axes. Only vectors with a Pearson correlation greater than 0.5 are shown.**

6.4.3 Regional patterns of decapod larval distribution in 2007

Throughout the sampling area temperature and salinity were vertically homogeneous from the surface to about 130 m, where the start of a thermocline was found. Therefore, a reference depth of 50 m was chosen as representative of the temperature and salinity fields between the surface and 130 m (Fig. 4). The Algarve region, located in the Gulf of Cadiz, showed higher temperature (Fig. 4a) and salinity (Fig. 4b) values than the other zones in this surface layer. The Sagres region and the southwest part of the Alentejo region had similar temperature (Fig. 4a) and salinity (Fig. 4b) values. Colder and fresher waters were found in the northeast part of the Alentejo region (Fig. 4).

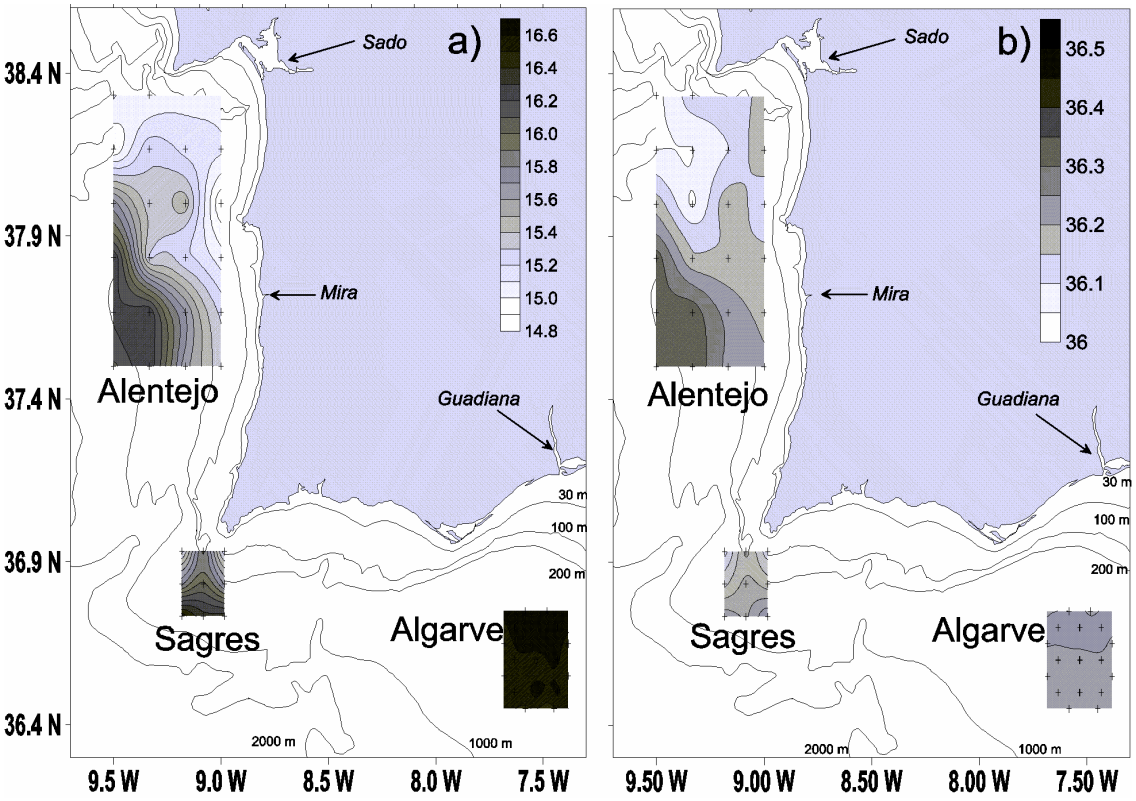


Figure 6.4: ***Physical data:*** **(a) Temperature (ºC), (b) salinity, pattern at 50 m depth in the three areas of sampling off the south coast of Portugal during the 2007 cruise.**

The temperature and salinity fields off Alentejo indicated the presence of two different water masses separated by a well defined frontal region aligned in the NW-SE direction (Fig. 4). The presence of this front in the surface layer can also be

recognized in satellite images extracted for the region (not shown) and by the clustering of the vertical profiles in two groups on the basis of the location of the sampling stations relative to the front above 150 m (Fig. 5), despite of a less clear segregation of profiles from stations within, and adjacent to, the front. A further distinction of the vertical profiles is that they showed a more clearly defined and stronger thermocline in the SW side of the front than in the NE (Fig. 5).

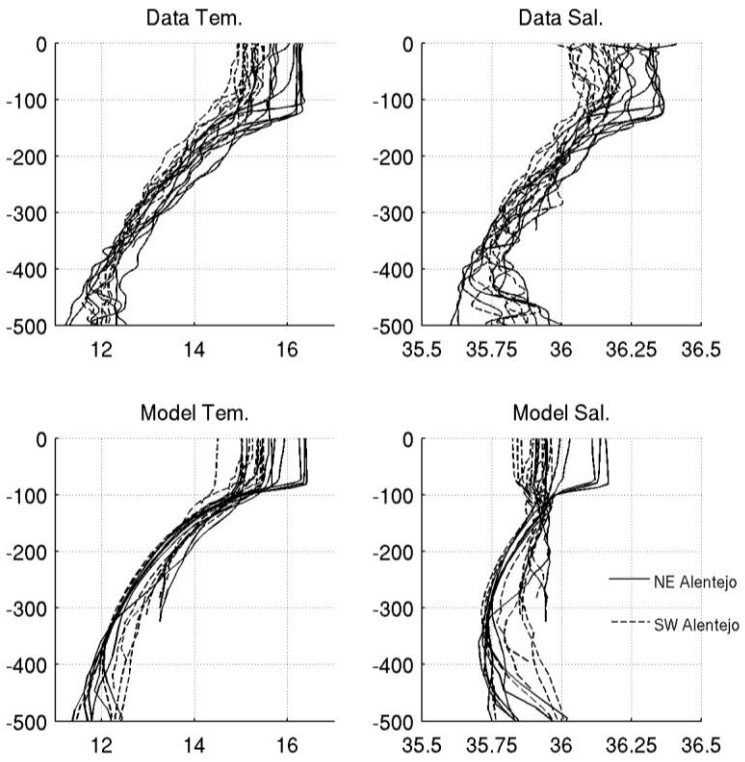


Figure 6.5: *Physical data:* Vertical profiles for *in situ* measurement (top) and model predictions (bottom) for temperature (ºC), and salinity fields in the Alentejo sampling area during the 2007 cruise.

Northerly upwelling favorable winds were predominant during most of January 2007, reaching higher intensity during the period of 21th to 27th of January, preceding the sampling in the Alentejo coast that took place between January 29th and 31st (Fig. 6b). Therefore, the front separating the offshore warmer and saltier waters from the colder and fresher waters closer to the coast can be interpreted as resulting from this upwelling event. This interpretation is supported by the model results, which reproduce many of the observed hydrological features. The vertical profiles generated

by the model for each station also show a segregation of the water masses at the surface and a decrease in salinity and temperature closer to the coast caused by the upwelled water (Fig. 5), as well as a frontal structure clearly developed by the 28th of January (Fig. 6a), although the predicted salinity values at the surface are lower than the ones recorded. The model also predicts some recirculation in the area with a northward flow offshore and a southward flow of coastal region (Fig. 6a, blue color in the temperature, salinity and velocity fields). This upwelling event may also be the cause for the clear difference observed in the zooplankton distribution between the two sides of the front, with much lower abundances observed inshore of the front (Fig. 2f).

The presence of this front and of the different water masses motivated the separation of the Alentejo region into two sub-regions, the Northeast Alentejo and the Southwest Alentejo, when analyzing the regional patterns of larval abundance in 2007. In this year peak abundance occurred off the coast of Alentejo and in the Algarve region. The species present also differed between the zones. A total of 24 taxa were identified in the Algarve, 27 in Sagres, 22 in the Southwest Alentejo and 30 in the Northeast Alentejo (Table 1). Abundance and diversity of Dendrobranchiata were highest in the Algarve region but very low in all sampling sites of the three other areas, from where they were sometimes absent (Fig. 2c). Results of the PERMANOVA indicated that there were no differences in the distribution and abundance in relation of the phase of the day ($p=0.93$). Larval composition differed between the regions sampled $p=0.0014$; Fig. 7). The Sagres and the southwest of Alentejo had similar species abundance and distribution ($p=0.57$), grouping together in the PCO plot, but the other areas were all different from each other ($p<0.04$ in all pairwise comparisons). The (differences in species composition resided mainly in the composition of crabs as *Monodaeus couchi* and *Goneplax rhomboides* (Figure 8 and 9) that were most abundant in the Algarve, whereas *Cancer* spp., *Carcinus maenas* and *Atelecyclus rotundatus* (Figure 10, 11 and 12) distinguished the Northeast Alentejo from the other areas (Fig. 7). Moreover, the last three species were absent from the southeast Alentejo.

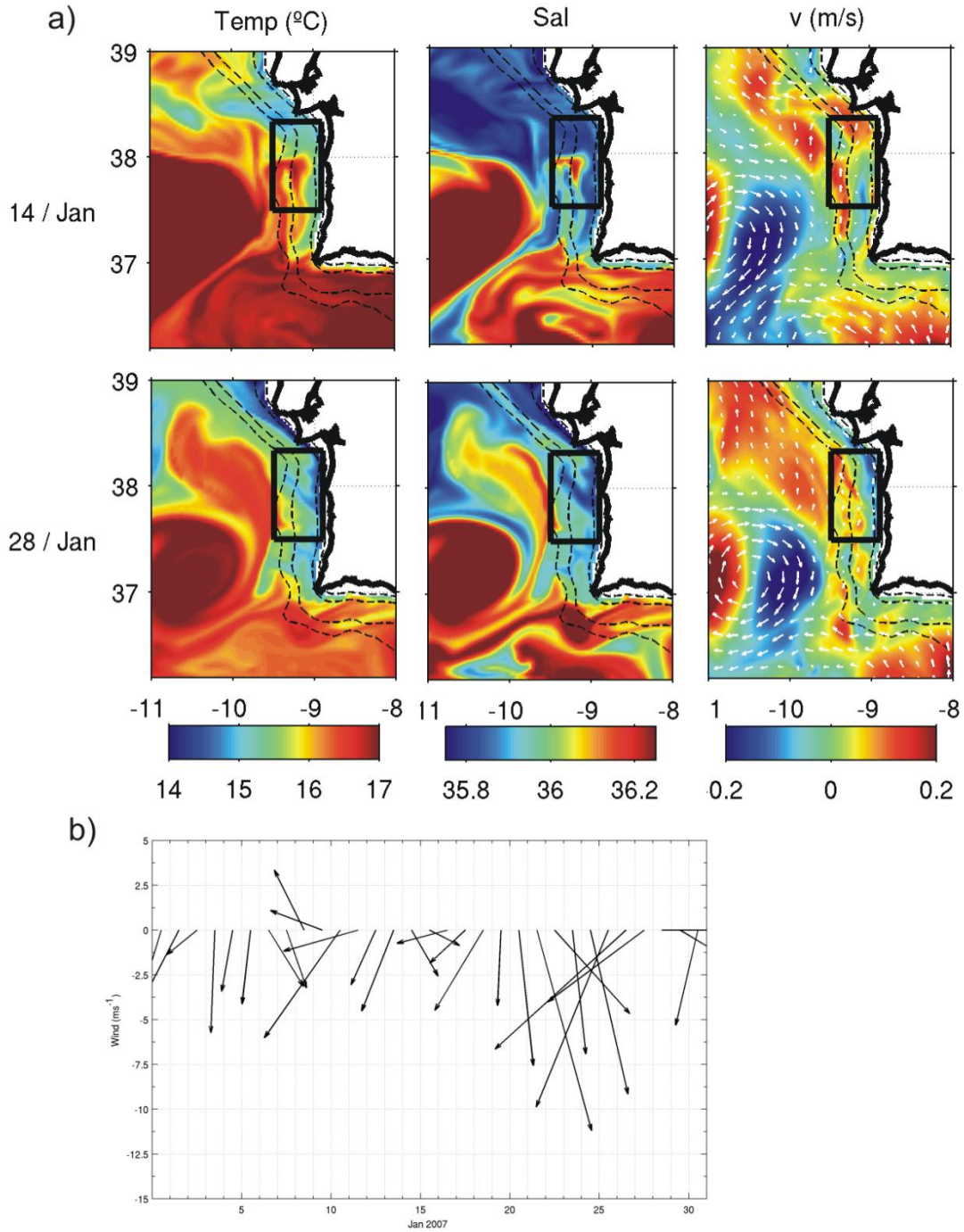


Figure 6.6: ***Oceanographic model*** **(top): Temporal evolution of simulated temperature (1st column), salinity (2nd column) and meridional component of the velocity (3rd column) fields from the 14th and 28th of January, 2007. The rectangle represents the region sampled during the cruise between January 29th to 31st. Positive and negative values of velocity indicate poleward and equatorward velocities, respectively.** ***Wind data*** **(bottom): Stick diagram of daily average of wind obtained from QuikSCAT during January 1st to 31st, 2007 in the Alentejo area (9.25W, 37.5N). Negative wind speeds means upwelling favorable (northerly) winds. Data were extracted from CERSAT (cersat.ifremer.fr) with 0.25º spatial resolution.**

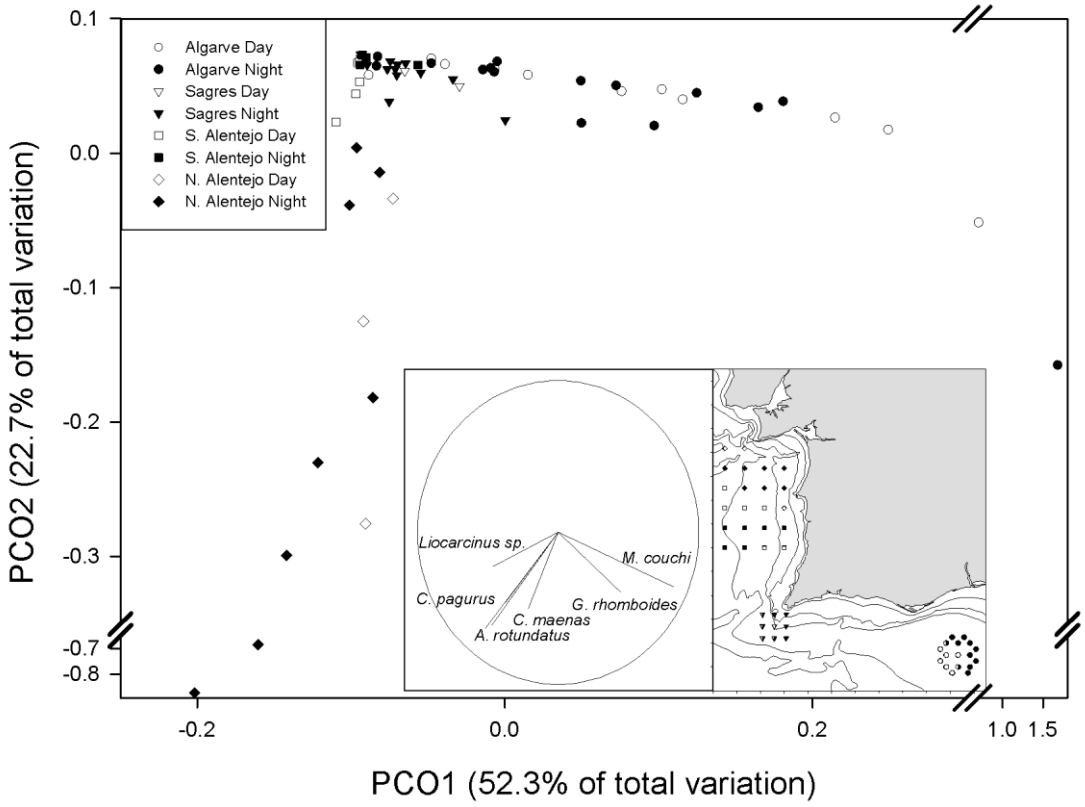


Figure 6.7: ***Principal Coordinate Analysis (PCO) comparing offshore decapod distribution between the different regions sampled during the 2007 cruise*. Inset map indicates sampling locations. Black and white triangles, circles and squares represent Algarve, Sagres, southwest Alentejo and northeast Alentejo sites, respectively. Vector length and direction indicate strength and sign of the relationship between the variable and the PCO axes. Only vectors with a Pearson correlation greater than 0.5 are shown.**

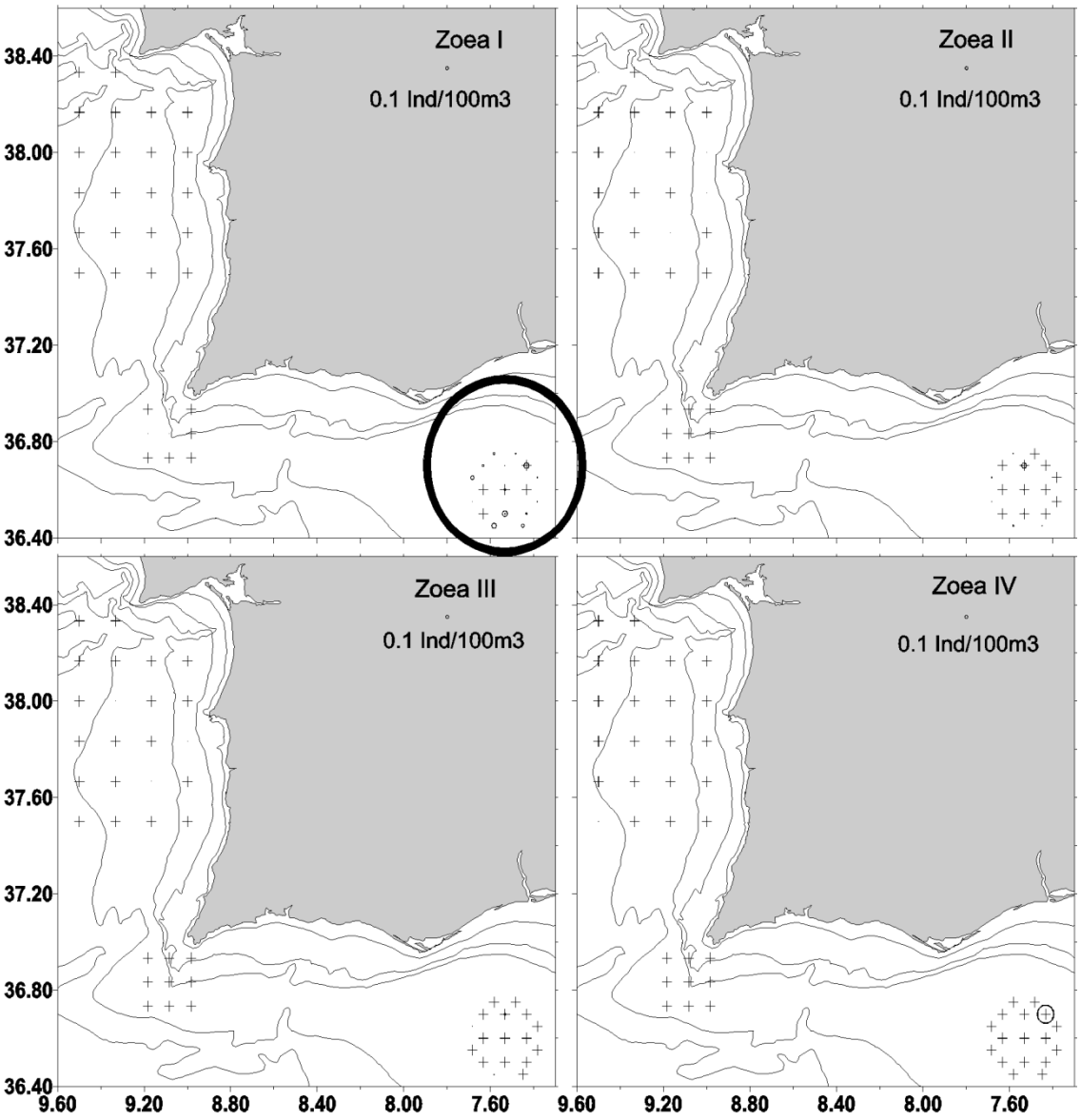


Figure 6.8: *Monodaeus couchi* abundances: Vertically integrated concentrations of *Monodaeus couchi* larvae in locations sampled in 2006 and 2007, for Zoea I to IV. The size of the circle represents larval concentration according to the scale shown in each panel (ind 100 m^{-3}). Sampling sites marked with a cross indicate absence of larvae from this species.

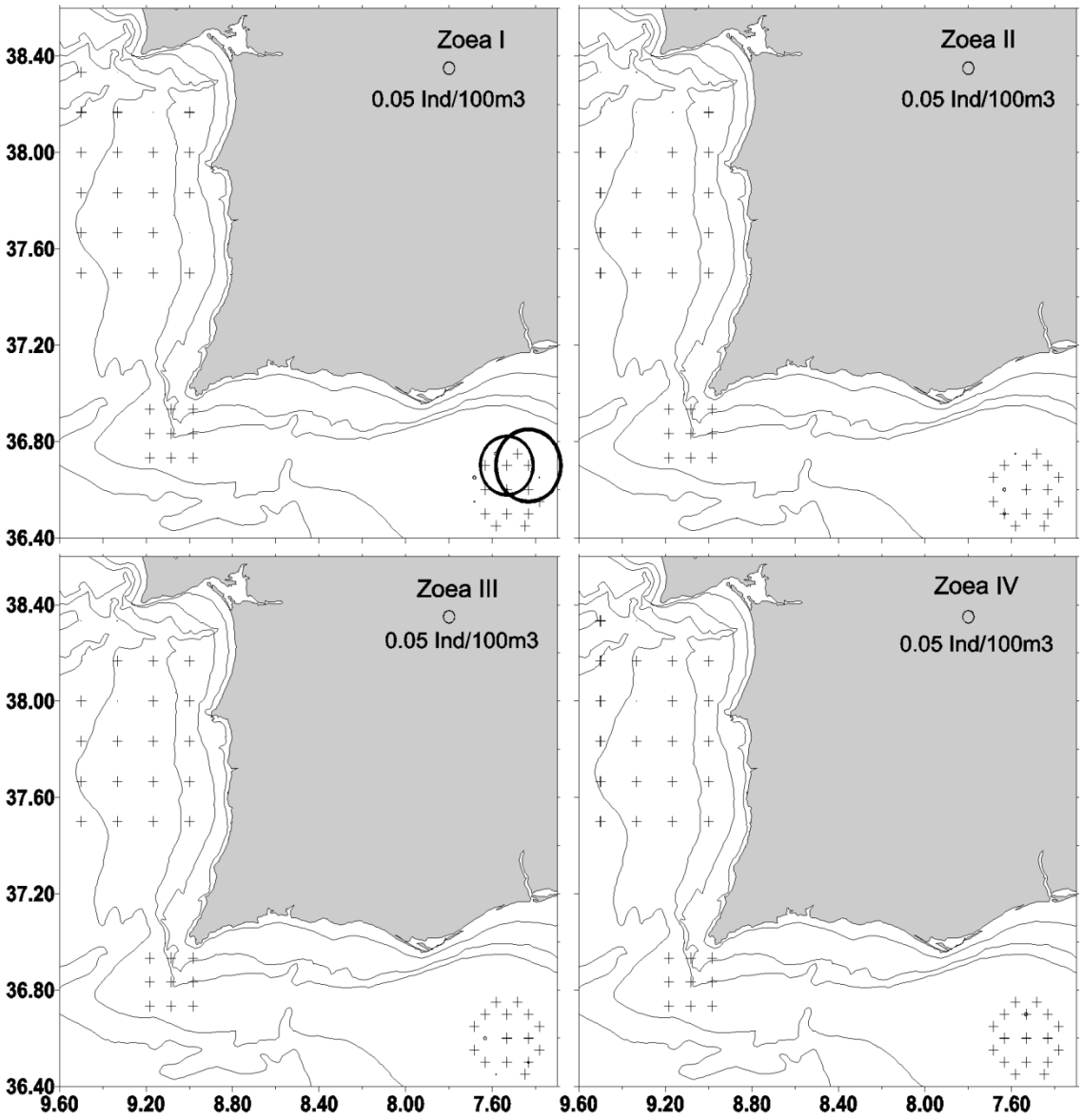


Figure 6.9: *Goneplax rhomboides* abundances: Vertically integrated concentrations of *Goneplax rhomboides* larvae in locations sampled in 2006 and 2007, for Zoea I to IV. The size of the circle represents larval concentration according to the scale shown in each panel (ind 100 m^{-3}). Sampling sites marked with a cross indicate absence of larvae from this species.

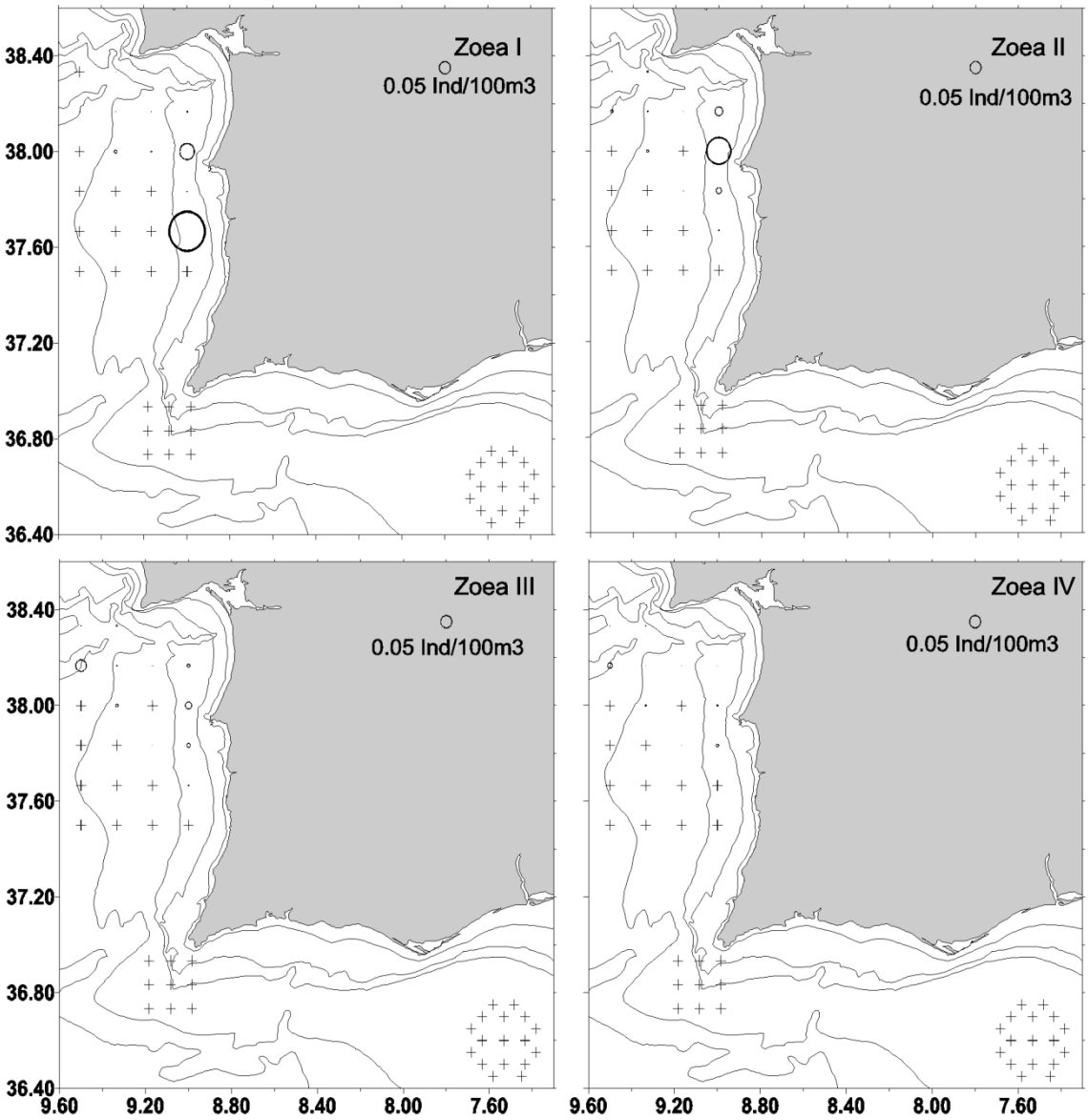


Figure 6.10: *Cancer* spp. abundances: Vertically integrated concentrations of *Cancer* spp. larvae in locations sampled in 2006 and 2007, for Zoea I to IV. The size of the circle represents larval concentration according to the scale shown in each panel (ind 100 m^{-3}). Sampling sites marked with a cross indicate absence of larvae from this species.

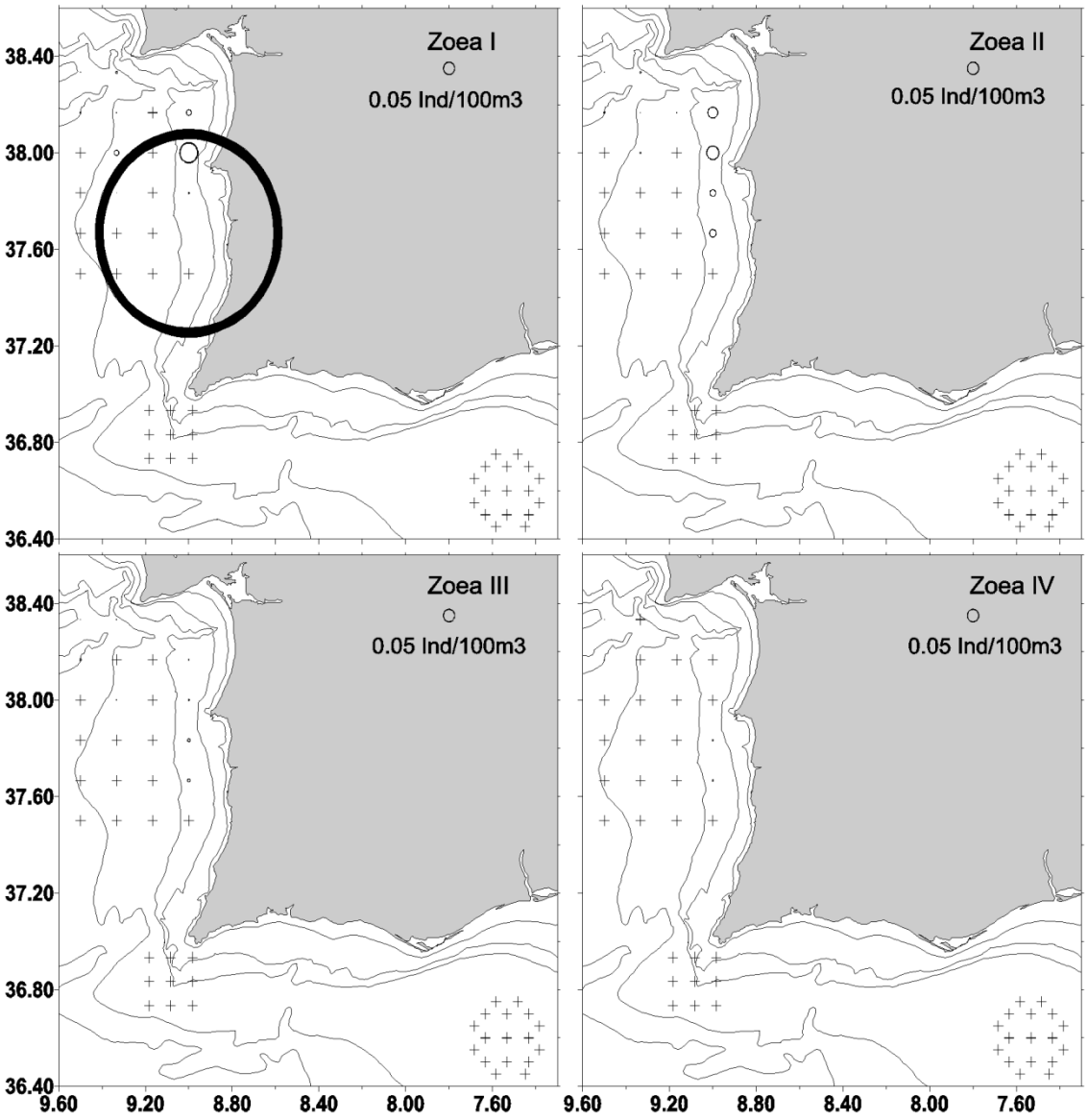


Figure 6.11: *Atelecyclus rotundatus* abundances: Vertically integrated concentrations of *Atelecyclus rotundatus* larvae in locations sampled in 2006 and 2007, for Zoea I to IV. The size of the circle represents larval concentration according to the scale shown in each panel (ind 100 m^{-3}). Sampling sites marked with a cross indicate absence of larvae from this species

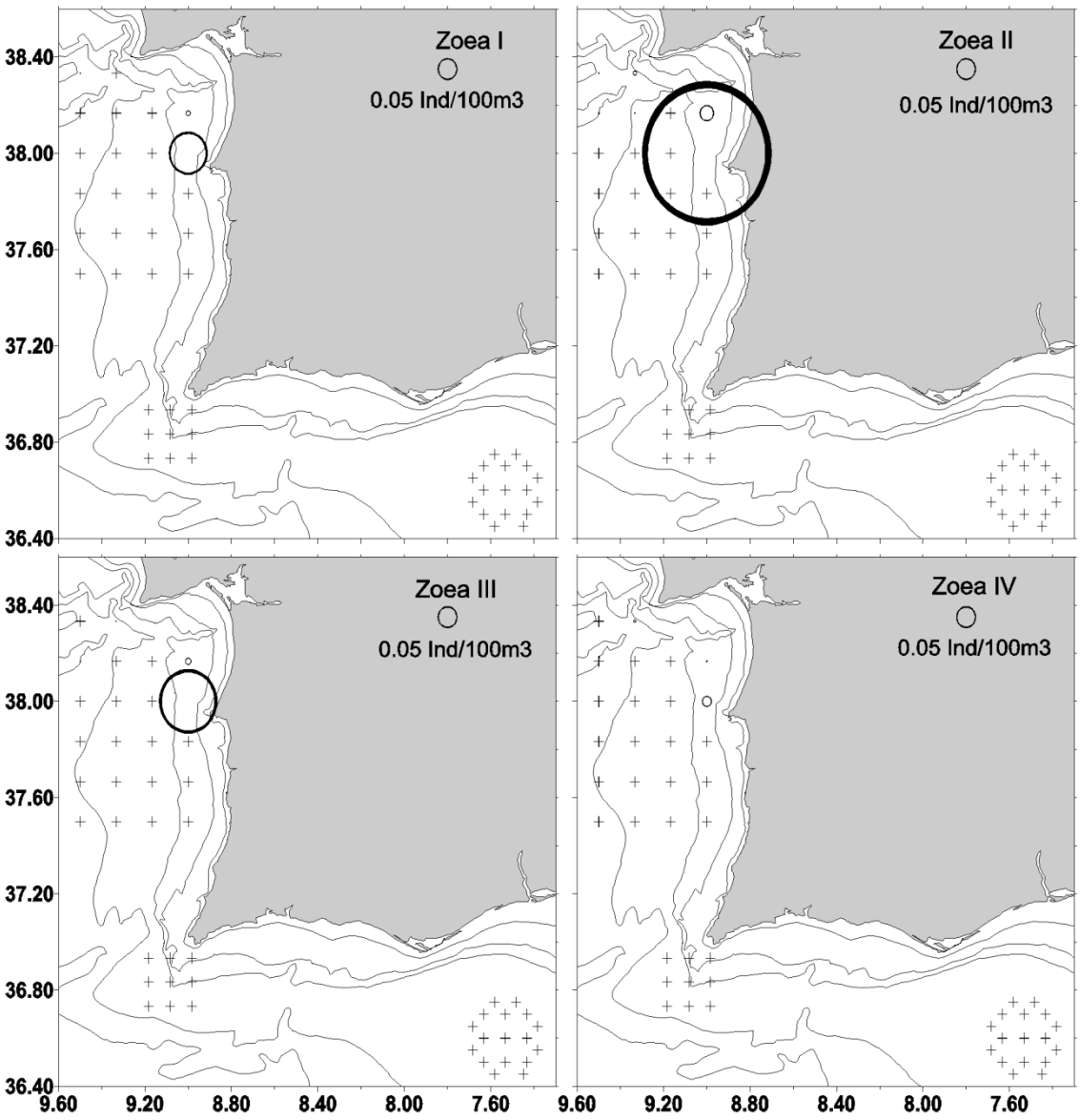
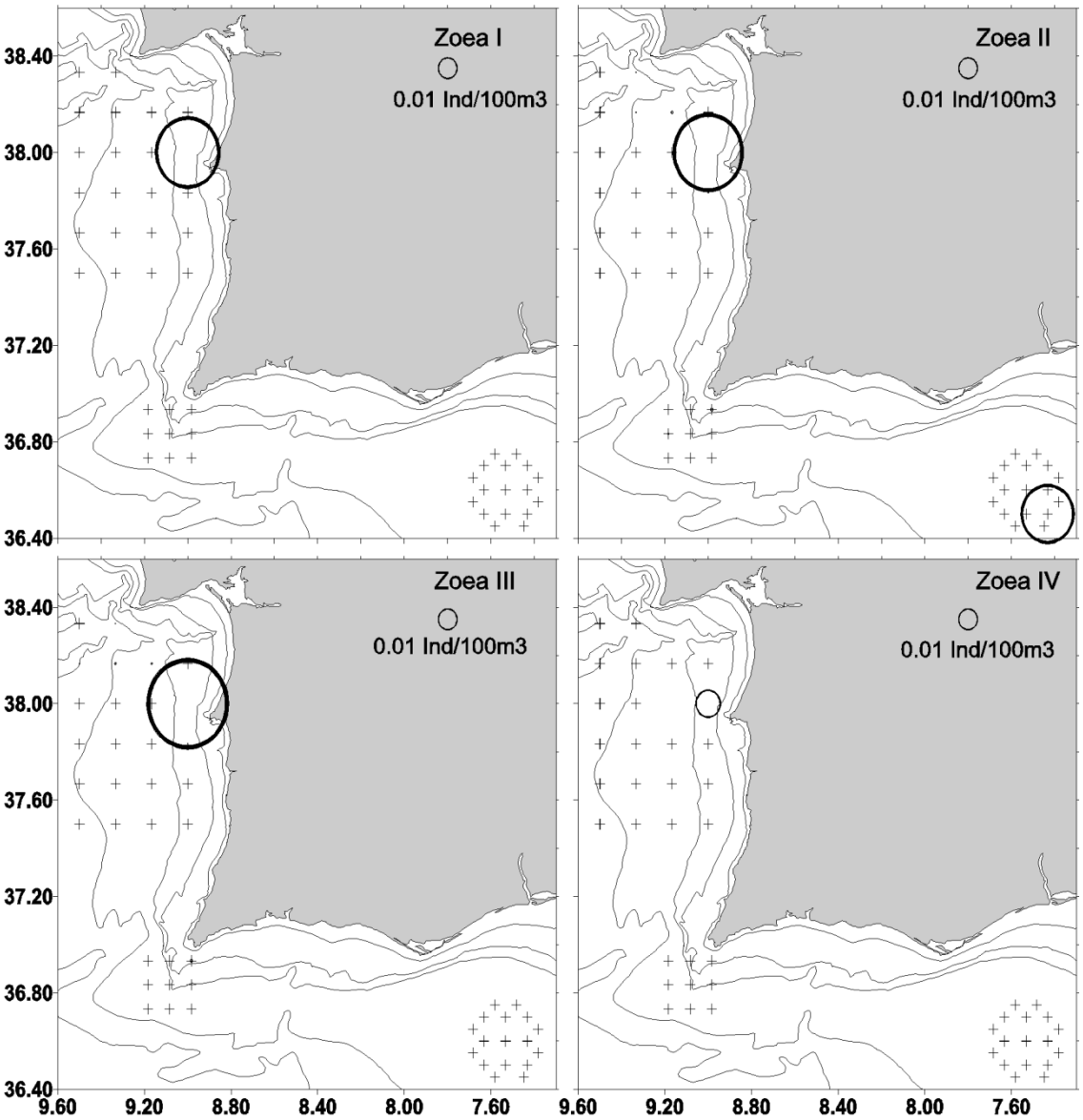


Figure 6.12: *Carcinus maenas* abundances: Vertically integrated concentrations of *Carcinus maenas* larvae in locations sampled in 2006 and 2007, for Zoea I to IV. The size of the circle represents larval concentration according to the scale shown in each panel (ind 100 m^{-3}). Sampling sites marked with a cross indicate absence of larvae from this species.



6.13: *Anapagurus* spp. abundances: Vertically integrated concentrations of *Anapagurus* spp. larvae in locations sampled in 2006 and 2007, for Zoea I to IV. The size of the circle represents larval concentration according to the scale shown in each panel (ind 100 m^{-3}). Sampling sites marked with a cross indicate absence of larvae from this species.

6.4.4 Inter-annual differences in decapod larval distribution in the Algarve region

Results of PERMANOVA indicated that there was a significant difference in the species composition and abundance in the offshore area of the Algarve between the two years of sampling (p=0.0039; Fig. 14) but there was no difference in larval distribution between day and night samples (p=0.37). A total of 18 taxa were encountered both

years, 9 were only found in 2006 and 8 only in 2007. The difference between the two years was explained by the high abundances of the crabs, *Monodaeus couchi* and *Goneplax rhomboides*, the Anomuran squat lobster *Munida* spp. and the shrimp *Solenocera membranacea* in 2006 whereas in 2007 abundances of *Sergia robusta* was much higher than in 2006. Additionally, *Pilumnus* spp., which was encountered in high abundance in 2006, was completely absent the following year (Fig. 14).

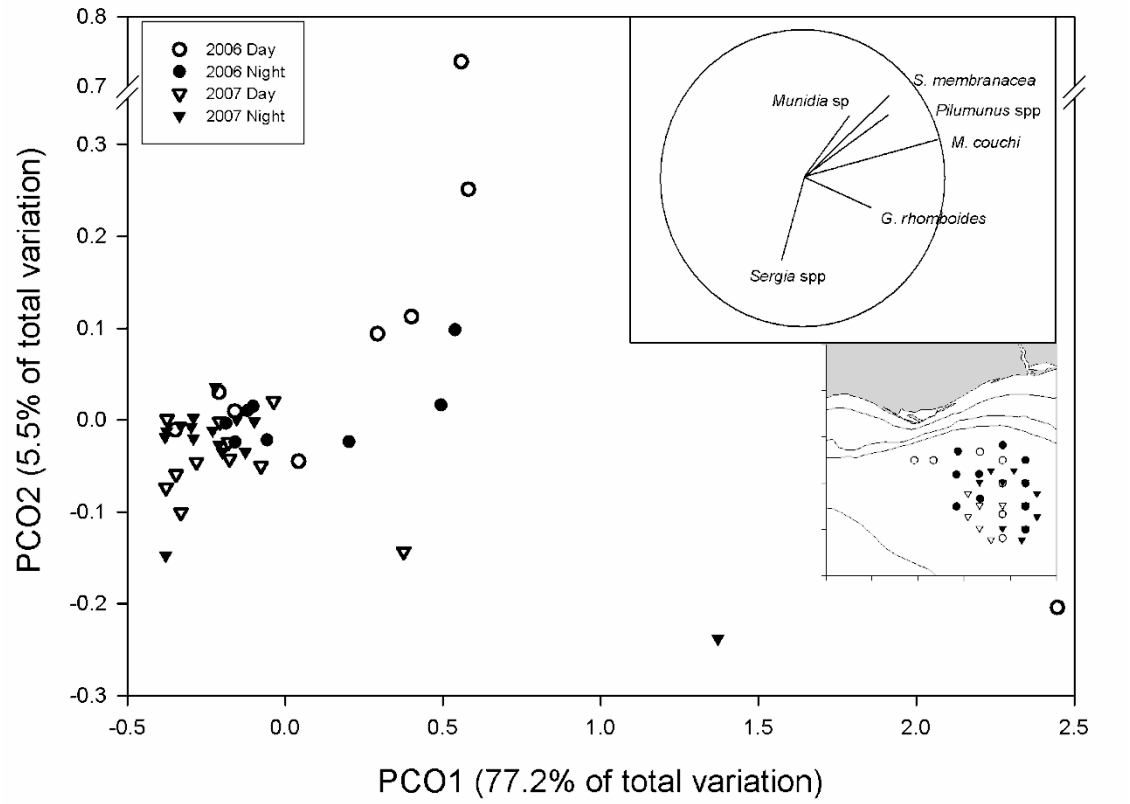


Figure 6.14: ***Principal Coordinate Analysis (PCO) comparing offshore decapod distribution between 2006 and 2007 cruises*****. Inset map indicates sampling locations. Black and grey triangles represent 2006 and 2007 sampling site, respectively. Vector length and direction indicate strength and sign of the relationship between the variable and the PCO axes. Only vectors with a Pearson correlation greater than 0.5 are shown.**

6.5 Discussion

Decapod distribution varied between years, distances from the coast and geographical area. Sampling sites located in coastal areas were dominated by estuarine species (e.g., *Carcinus maenas, Nepinnotheres pinnotheres, Diogenes pugilator*). Regardless of

areas or year of sampling, the composition and abundance of decapod larvae was independent from the phase of the day. Since samples were taken between the surface and a maximum of 300 m and integrate the entire water column, it can only be deduced that the larvae are remaining between these depths and that, even if vertical migration takes place, they do not migrate below that point. These results are not surprising, as the diel vertical migration performed by most species is encompassed within this depth range (reviewed by Queiroga and Blanton 2005, Table 4) and suggest that the nocturnal vertical migration behavior that is generally depicted by decapods larvae in the ocean was restricted to this depth level.

All *taxa* identified to species level can be categorized into two groups regarding the geographical distribution of adults (d'Udekem d'Acoz 1999; Manning and Holthuis 1981; Zariquiey Alvarez 1968): i) species with known geographical limits located somewhere between Norway and Mauritania (e. g. *Cancer* spp., *Nephrops norvegicus*, *Crangon crangon*, *Carcinus maenas* and *Polybius henslowii*; ii) species with known geographical limits located somewhere between Norway and South Africa (e. g. *Monodaeus couchii*, *Diogenes pugilator*, *Parapenaeus longirostris* and *Athanas nitescens*). Some of the species in the second group have populations in the tropical West African coast, while others reached an anti-tropical distribution presumably by latitudinal submergence (Briggs 1974; Hedgepeth 1957). All these species except *C. maenas* also occur in the Mediterranean. *Taxa* identified to genus level include species belonging to these two categories as well as tropical species. In neither case these genuses include only tropical species. Therefore, the larval assemblage found in the south and southwest coasts of Portugal during the two winters sampled can be considered as typical of the Lusitanian Province, Mediterranean-Atlantic Region, with some influence from more boreal species (Briggs 1974).

For both years, the abundance of larvae was relatively low. Two factors may contribute to the relative paucity of the larvae. The first is a seasonal effect. Indeed, in a spring campaign over the Portuguese western shelf, larval abundances was one to two orders of magnitude higher that what was recorded in the present study (Table 2,

dos Santos et al. 2008). Most species in the area reproduce primarily during the spring and summer months, and higher overall abundances are expected during this period. However, a previous study by dos Santos (1999) found that larvae of *Carcinus maenas*, *Ebalia* spp., and species from the sub-family Polybiinae (e.g. *Liocarcinus* spp., M*acropipus* spp., and *Polybius henslowii*) were primarily found during the winter, and occurrence of *Gennadas elegans, Nephrops norvegicus* and *Palinurus elephas* larvae was limited to winter months. In this sense, decapod larvae composition in the winters of 2006 and 2007 was similar to that observed by dos Santos (1999). The second factor is that most sampling stations were located over the outer shelf and the slope. Therefore, sampling may have missed the large abundances of coastal and estuarine species that several studies have recorded over the inner shelf in eastern margins of the North Atlantic and the North Pacific (dos Santos et al. 2008; Morgan et al. 2009; Queiroga 1996).

During the 2006 campaign in the Algarve, differences in species composition were observed between coastal and offshore areas. The former was dominated by coastal caridean shrimps, while the latter was dominated by open ocean and deep sea species such as the shrimps *Sergestes* spp. and *Solenocera membranacea* and the brachyuran crab *Monodaeus couchi*. Species composition, both coastal and offshore, was similar to what has been previously observed in the Algarve under winter conditions (dos Santos 1999). Another important factor contrasting onshore with offshore waters was the presence of estuarine species associated with the river outflow. Abundance and diversity of the eastern part of the Algarve sampling region was highly influenced by the discharge from the Guadiana river. The most coastal sampling sites in that area was dominated by estuarine species which undergo part of the larval cycle offshore (*e.g. Pilumnus* spp., some *Liocarcinus* spp.).

In 2007, decapod larvae composition was similar between the SW Alentejo and the Sagres regions, which differed from the Algarve and NE Alentejo regions. While the most abundant species in the Algarve were *Monodaeus couchi* and *Goneplax rhomboides*, the NE Alentejo was dominated by brachyuran crabs, namely *Carcinus*

maenas, *Cancer* spp. and *Atelecyclus rotundatus* and by the anomuran *Anapagurus* spp.. The latter four taxa, encountered in most sampling locations in the NE Alentejo, were completely absent offshore of the front. In addition, higher larval abundances were observed over the continental shelf (100-200m depth) indicating that larvae are probably being retained in this area in spite of the prolonged upwelling event that was observed. Indeed, the wind and hydrographic data of 2007, as well as the model interpretation, indicate that upwelling favorable winds originated the frontal structure off the Alentejo coast, separating the upwelled waters with northern origin from the offshore warmer and saltier waters with subtropical origin.

The presence of coastal species in the NE Alentejo (*Carcinus maenas*, and *Anapagurus spp.*) greatly contributed to differentiate the assemblages across the front. Higher larval abundances observed in the most coastal sampling location over the shelf indicated that coastal species were retained onshore (Figure 12 and 13). Such retention during upwelling events has largely been documented (e.g.: dos Santos et al. 2008; Morgan et al. 2009; Queiroga et al. 2007) and has been linked to different kinds of depth-regulation behavior. Nevertheless these are coastal species and our sampling design therefore does not permit a full comprehension of larval dispersal as higher abundances are expected onshore and not on the external part of the continental shelf where our sampling was performed. This is however not the case for species which adults commonly inhabit the external area of the continental shelf such as *Cancer* spp. and *Atelecyclus* spp.. The presence of adult populations over the shelf most likely resulted in supply of larvae to this general area. Offshore larval abundance of *C.* spp. and *Atelecyclus* spp. in the NE Alentejo region increased with stage (Figure 10 and 11) indicating that a small portion of the larvae were not retained over the shelf but were carried beyond the shelf break by the upwelled waters.

Because of coastal topography, recirculation occurs in the Lisbon and Setúbal bays, in the north of the Alentejo region, especially in response to upwelling-favorable winds (Moita et al. 2003). At a larger scale, the ocean of the southwest Iberian region is characterized by the presence of two water masses separated by a W-E frontal zone

located between 38-39ºN (Peliz et al 2005). This frontal zone separates the colder and fresher waters of the subpolar branch of the Eastern North Atlantic Central Waters (ENACW) form warmer and saltier waters usually associated to the subtropical branch of the ENACW, which originates in the Azores region, and connects with the Gulf of Cadiz and the southwest region of the Iberian Peninsula through the northern branches of the Azores system of currents (Peliz et al 2007). Intrusions of these waters across the W-E front are frequently observed, and it has been hypothesized that these intrusions are associated with the northward deflections of branches of the Azores current in the vicinity of the Cape St. Vincent, in the Sagres region (Peliz et al. 2005). The model results reproduce to some extent these patterns, showing a coastal southward flow and a northward flow offshore over the sampling area, as well warm eddies derived from the Azores current further south and offshore (see Fig. 6). The northward flow of warm water west of the Alentejo predicted by the model between 37.5 and 39.0^{o} N, which was deflected offshore by the upwelling event, may be a reflection of this. This interpretation is also supported by the observed hydrological fields that were very similar in the Sagres and the southwestern Alentejo areas. These mechanisms could underlie the similarity of the decapod larval assemblages of the SW Alentejo and of the Sagres regions (about 2/3 of the species were common to both areas), suggesting that the larvae present in the Sagres region were probably brought northward toward the southwestern part of Alentejo. Moreover, some input of larvae from northern areas such as the Lisbon bay might have impacted the decapod larval composition of the NE Alentejo.

Zooplankton abundance (Fig. 2f) was higher in the SW Alentejo and in the Algarve than in the NE Alentejo, where very low levels were found in some stations. The low abundance of zooplankton in the NE Alentejo contrasts with the high abundance of decapod larvae in this region, especially in the more coastal sampling sites. This distribution of the zooplankton can be explained by the upwelling event assuming a supply of recently upwelled water with low biomass of phyto- and zooplankton. While distribution of holoplanktonic organisms depends exclusively on circulation patterns and water productivity, decapod larvae abundances depend on production from

benthic dwelling females, which may decouple the abundances of both planktonic groups.

As currents highly influence larval distribution, time evolution of the circulation patterns might explain the differences observed in species composition of offshore species in the Algarve area between the two years of sampling. The main difference observed is the lower abundance of shrimp larvae caught in 2007. Indeed, excluding *Sergia robusta*, all other species caught in 2006 were only found at very low densities the following year (Table 1). Similarly, benthic species abundant in coastal waters in 2006 were almost not encountered in 2007 such as the crabs *Monodaeus couchi*, *Goneplax rhomboides* and *Pilumnus* spp.. Those inter-annual differences may be due to various factors, including variations in current patterns, like presence of meddies, variations in adult reproduction time and larval duration.

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Chapter 7

Concluding Remarks

7.1 Concluding Remarks

Decapod crustaceans have evolved numerous mechanisms that optimize survival and growth throughout embryonic and larval development. During the early phases of the life cycle, the energetic demands are very high. Indeed, essential nutrients are required for proper organ development or successful metamorphosis. For this reasons, the period of suboptimal food conditions at any point of the life cycle can significantly affect the subsequent stages such as larvae and post-settled individuals. Since embryonic development is lecithotrophic and food might not be readily available immediately following birth, it can be assumed that the feeding status of females during vitellogenesis will influence offspring survival (Giménez 2006, 2010; Pechenik 2006). As inter-specific variability in fatty acid (FA) profiles are the results of different ecological niche and feeding regimes (Rosa et al. 2007a), it is likely that differences in habitat or inter-annual variations in food conditions can result in within-brood variations in embryonic biochemistry. The results of chapter 2 further indicate that there is also, at least in *Nephrops norvegicus*, a high variability in FA composition between embryos of a single clutch. This differential female investment into the embryos and/or differential catabolism of yolk reserves by the embryo can explain why in the laboratory there is great within-clutch variability in larval survival. Suboptimal post hatching environmental conditions will further affect developing larvae (reviewed by Giménez 2006) and those that had lower energetic embryonic input may not be able to successfully reach the following larval stage, even though short term survival to suboptimal food conditions can still be possible (e.g.: Calado et al. 2010; Gebauer et al. 1999; Giménez and Anger 2003, 2005; Ritar et al. 2003b; Smith et al. 2004).

Regardless of the quality of maternal investment, and because of the unpredictable nature of the open ocean, larvae demonstrate significant plasticity in prey preference, feeding on lower size and nutritional quality prey when adequate ones are absent (chapter 3). This shift in diet will also be accompanied by physiological adaptations, such as an increase in digestive enzymes activity (like protease activity increase in starved first-stage *N. norvegicus* larvae; chapter 4). Additionally, an increase in gut evacuation time (GET) can also be expected as it has been observed in many decapod species (Jones et al. 1997). Both an increase of enzyme activity and GET will allow

larvae to maximize nutrient assimilation in suboptimal food conditions (Kumlu 1999). Once food conditions recover, prey quality and quantity being adequate again, feeding rate will increase in order to recover from a starvation period, such as it is observed in stage I and II of *N. norvegicus* in chapter 3. However, if starvation occurs over an extended period of time, and the larvae will not be able to recover and successful development will not occur (Anger and Dawirs 1981). Indeed, extended period of starvation leads to damages to the hepatopancreatic cells and prevents the accumulation of lipids regardless of the quantity and quality of available food (Anger 2001). The larvae will not be able to metamorphose to the next stage and death will eventually occur (Anger 2001). As observed with amylase activity in starved newly hatched *N. norvegicus*, sustained starvation can result in a failure to trigger specific digestive enzymes, which could translate into a deficiency in nutrients essential to successful development and molting to the next larval stage. Since carbohydrates play an important role in the molting process (Luo et al. 2008), a failure to activate amylase activity before reaching the end of larval stages, would probably negatively impact metamorphosis (chapter 3).

In addition to food condition, predation pressure will also affect larval survival throughout development. In order to avoid predation, one possible strategy is to perform a different type of DVM than their predators (Han and Straskraba 2001; Pearre 2003). *Monodaeus couchi* represents a good example of such species, as they were observed to perform reverse diel vertical migration off the coast of Portugal in 2006 and 2007 (chapter 5). Such behavior however will also translate into lower prey availability to the organism performing that uncommon type of migration (Irigoien et al. 2004). Nevertheless, depending on the feeding strategy, if female nutrient input into the yolk reserves is sufficient, the resistance to starvation will increase and the larvae can develop successfully in an environment with lower predation pressure. Nevertheless, unless the species is fully lecithotrophic, larvae will have to feed eventually, and as larval size increases, prey preference will tend to shift towards larger prey (as observed in *N. norvegicus* in chapter 3) and possibly to more energetic food items.

Regardless of the vertical migration strategy, the positioning in the water column as well as survival will eventually influence dispersal and recruitment as larvae will be transported by depth varying currents (reviewed by Queiroga and Blanton 2005). Global circulation patterns will dictate general trends in larval transport but short term variations in environmental conditions, such as changes in atmospheric conditions, will result in temporal difference in local larval composition and abundance. The low numbers in larval abundances observed in chapter 5 and 6 can be explained by the time of sampling (winter) combined, in the case of the Alentejo region, with the small size of the shelf in that area. Indeed, dos Santos and Peliz (2005) indicated that very few *N. norvegicus* larvae were encountered in the region despite the large adult population and similar results were observed for *M. couchi*. Modeling studies indicated that only a small proportion of larvae hatching in the Algarve are likely to reach the Alentejo region. Marta-Almeida et al. (2008) modeled the potential dispersal of *N. norvegicus* larvae off the coast of Algarve during the winter based on the estimated area of the adult populations and different vertical migration behavior scenarios of the larvae. Since no difference in decapod abundance is observed between day and night samples (chapter 5) and *M. couchi* exhibited reverse DVM between the surface and 200 m (chapter 6), it can be assumed that DVM behavior of decapods larvae of the Portuguese coast during the winter are comparable to either the DVM200 (diel vertical migration up to 200 m) or U200 (uniformly distributed in the top 200m) simulations of the model (Marta-Almeida et al. 2008). For both type of behaviors, the results indicated that most of the larvae hatching in the Algarve were retained in that area, close to the coast, in the area where decapod abundance was highest in our samples. Indeed, according to the model, very few larvae are transported towards the coast of Alentejo. This could explain the lower decapod larvae abundance encountered in that area in chapter 5, especially if the adult populations are reduced in this area due to the small width of the shelf. Those results are consistent with dos Santos and Peliz (2005) where *N. norvegicus* larvae were not observed in the Alentejo region despite the presence of an important population on the slope. Similarly, in our study, *N. norvegicus* larvae were only collected in the Algarve.

Unpredictable events can either transport larval to a new habitat to colonize, or export it to an environment where settlement and therefore survival will not be possible. Such a case is illustrated by the upwelling event that was occurring off the coast of Alentejo in the winter of 2007, causing the area to be divided by a front. Larval composition on each side of the front is clearly different, with a retention occurring for coastal species in the NE area (chapter 6). The presence of adult populations over the shelf most likely resulted in constant supply of larvae in the area, explaining the higher larval abundances in the most coastal locations of our sampling plan. Interestingly, the NE part of the Alentejo, influenced by upwelled chlorophyll poor waters, show lower zooplankton abundance. Indeed, the higher offshore abundance of zooplankton in the SW Alentejo, is not consistent with the higher decapod larvae abundances observed in the NE Alentejo. Differences in life cycle, in the benthic and localized origin of the larvae contrasting with the largely unbounded production processes of holopkantonic organisms, as well as in behavior, most likely explain the observed differences.

7.2 Future works

The two species used in the present work are good models to better understand the early life biology of deep-sea species and the evolution of adaptations to deep-sea habitats. Both species occupy a range of depths from shallow shelf habitats to hundreds of meters (more than 1000 m in the case of *M. couchi*). *M. couchi* is a fairly widespread species and its larvae are encountered at high densities at some places, but the remoteness of the deeper adult habitats and the lack of commercial interest have translated into little knowledge of its biology. In contrast, *N. norvegicus* is a much better studied species, especially in northern latitudes where adults inhabit shallower waters. Its high commercial value facilitates the acquisition of benthic juveniles and adults from the fisheries. On the other hand, the decrease in population size as a result of overfishing, combined with low reproductive output and a wide offshore distribution, result in low number of larvae present in the plankton off the Portuguese coast and therefore hindered the study of its planktonic phase. In fact, during both oceanographic campaigns of 2006 and 2007 only 4 larvae of *N. norvegicus* were

encountered each year (see Table 6.1). These abundances are much lower than those encountered previously by dos Santos and Peliz (2005). In their study, reporting data collected between 1987 and 2001, annual variations in larval abundance were observed, with a slight overall decrease over time. This reduction in larval abundance is however negligible when compared with abundances recorded in our study, which were about two orders of magnitude lower than those recorded by dos Santos and Peliz (2005). Despite the variety of sampling techniques used in both this study and Santos and Peliz (2005) it is evident that the reproductive output of this population has decreased as net differences alone cannot account for the observed decrease.

For this reasons, and as performed in the present thesis, further works intending to increase the knowledge of offshore benthic decapods should therefore focus a variety of species. Different species could therefore be used to serve as models to answer specific questions. For instance, a better understanding of oocyte production and maternal investment could be obtained from commercially harvested larger species, while laboratorial studies could focus on smaller species with shorter embryonic development and higher reproductive output. Studying ovigerous females maturating in the field and in the laboratory may help us to better understand vitellogenesis and how the feeding status of the female influences nutrient allocation into produced embryos. In addition to maternal investment, variations in environmental conditions within the brooding chamber (e.g.: oxygen concentration, temperature, salinity, etc.) will cause variations in embryonic metabolism, resulting in differential use of yolk reserves (Brante et al. 2003), and eventually leading to asynchronous larval hatching (Eriksson et al. 2006; Fernandez et al. 2003). The exact cause of inter-individual and within-brood variability in embryonic biochemistry, as well as the resulting effect on larval development and survival, still requires further investigation.

Most larval behaviors, either endo- or exogenously cued, are extremely difficult to study *in situ*. In the present thesis, laboratorial hatching of *N. norvegicus* proved to be difficult and culture of this species to juvenile stage was unsuccessful. Most works on this subject date back in the 70's and 80's (e.g.: Anger and Puschel 1986; Figueiredo and Vilela 1972) and to date larviculture of this species is still in its experimental phase. Hatching and culturing healthy larvae in the laboratory is therefore needed. Potential

reasons for this failure include 1) the stress induced to females during capture and transport, 2) unsuitable nutritional quality of the prey provided (*Artemia* sp. nauplii) or 3) the morphology of the larvae itself, which possess large spinous processes along their body that are prone to injury upon contact with the walls of the culture chamber, causing potential infections. While optimizing the culture of *N. norvegicus*, an economically important species, is critical, more suitable candidates species, with higher reproductive output, should be selected to get a better global understanding of larval behavior of offshore benthic decapods. Laboratorial studies should investigate the responses of different larval stages to changing environmental conditions, mainly temperature, pressure and light, but also food availability. Long term effect of food deprivation or ingestion of suboptimal prey, as well as inter-individual variability, still requires further studies. In that sense, the use of biochemical tools allowing the evaluation of the physiological mechanisms and energetic state of reproducing females, embryos or larva (in some cases even at the individual level) holds many promises. Nevertheless, a full understanding of the processes regulating inter-individual and within-brood variability in biochemical profiles of embryos and larvae is needed, especially considering the ever-growing importance of the use of biomarkers in the study of populations (Dalsgaard et al. 2003).

A full understanding of the early life traits of offshore benthic decapods will therefore only be achieved through a multi-disciplinary approach including: 1) field sampling and oceanographic modeling to understand general larval behavior and dispersal, 2) small scale laboratory experiments to test individual larval responses, development and survival to changing environmental conditions; and 3) biochemical analyses to understand physiological adaptations of both embryos and larvae. Because of the carry-over effect in response to suboptimal environmental conditions that was observed in many decapod larvae, a holistic approach in the study of early life biology (starting at the embryo and finishing with juveniles) is advocated. Indeed, like it is the case in this thesis, until recently, many studies focus on a single life phase, if not a single stage, when repercussions of suboptimal conditions often significantly affect the individual later in life.

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